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RELEASE AND ACTIVITY OF ACE INHIBITORY PEPTIDES FROM PEA AND WHEY PROTEIN: FERMENTATION, *IN VITRO* DIGESTION AND TRANSPORT

VRIJSTELLING EN ACTIVITEIT VAN ACE-INHIBITORISCHE PEPTIDEN UIT ERWTEN- EN WEI-EIWIT: FERMENTATIE, *IN VITRO* VERTERING EN TRANSPORT

door

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VOORWOORD

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Vanessa

23 mei 2003

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Notation index

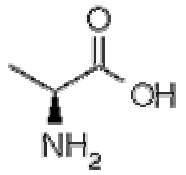
% (v/v)	percent volume over volume
% (w/v)	percent weight over volume
% (w/w)	percent weight over weight
AA	amino acid
ACE	angiotensin I converting enzyme
ATO	Instituut voor Agrotechnologisch Onderzoek / Institute for Agronomical and Technological Research
ATP	adenosine triphosphate
BAP	biologically active/bioactive peptide(s)
BSA	bovine serum albumin
BW	body weight
Caco-2	human caucasian colon adenocarcinoma cells
Caco-2 Bbe	human caucasian colon adenocarcinoma morphologically homogeneous, brush border expressing cells
CFU	colony forming units
CMP	casein glycomacropeptide
CN	casein
CPP	casein phosphopeptides
C-terminal	carboxy-terminal of protein / peptide
CVD	cardiovascular disease(s)
Da	dalton
DASH	dietary approaches to stop hypertension
DBP	diastolic blood pressure
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
E/S	enzyme over substrate ratio
EDTA	ethylenediaminetetraacetic acid
f()	fragment ()
FAP	2-furanacryloyl-phenylalanine
FAPGG	2-furanacryloyl-phenylalanyl-glycyl-glycine
FDA	Food and Drug Administration
FMOC	9-fluorenyl-methoxycarbonyl
FOSHU	food for specified health use
FUFOSE	Functional Food Science in Europe
g	gravity

GRAS	generally recognised as safe
GST	glutathion-S-transferase
HBSS	Hanks' balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HHL	hippuryl-histidyl-leucine
HPLC	high performance liquid chromatography
IC_{50} (mg/ml or mg P/ml)	50% inhibitory concentration (expressed as mg product per ml or mg protein per ml)
Ig-Hc	immunoglobulin - heavy chain
Ig-Lc	immunoglobulin - light chain
ILSI	International Life Science Institute
I_{sc}	short circuit current
k_{cat}	catalytic constant (expressed as $time^{-1}$)
K_m	Michaelis Menten constant (expressed as a concentration)
LHRH	luteinising hormone releasing hormone
LMG	Laboratory of Microbiology of Ghent (Culture collection)
LMW	low molecular weight
MALDI-TOF	matrix assisted laser desorption ionisation - time of flight
MAP	mean arterial blood pressure
max.	maximal
MES	2-morpholinoethanesulfonic acid
min.	minimal
MRS	de Man, Rogosa, Sharpe
MS	mass spectrometry
MWCO	molecular weight cut off
NCBI	National Center for Biotechnology
NLEA	Nutrition Labelling and Education Act
N-terminal	amino-terminal of peptide/protein
PUFA	polyunsaturated fatty acids
RP-HPLC	reversed phase - high performance liquid chromatography
SBP	systolic blood pressure
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SE	standard error
SHIME	simulator of the human intestinal microbial ecosystem
SHR	spontaneously hypertensive rat(s)
TCA	trichloroacetic acid
TEER	transepithelial electrical resistance
TFA	trifluoroacetic acid
TNBS	2,4,6-trinitrobenzenesulfonic acid
U	units
UTI	units of trypsin inhibitor

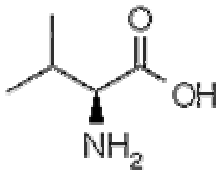
UV/VIS	ultraviolet / visible light
WHO	World Health Organisation
WKY	Wistar Kyoto rat(s)
YPD	yeast extract, bacteriological peptone, D-glucose
α-LA	α-lactalbumin
β-LG	β-lactoglobulin

Amino acids : structure and code

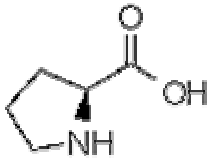
Nonpolar (hydrophobic)



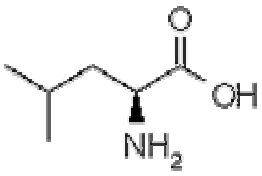
alanine (**Ala, A**)



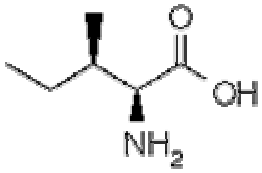
valine* (**Val, V**)



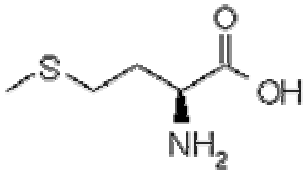
proline (**Pro, P**)



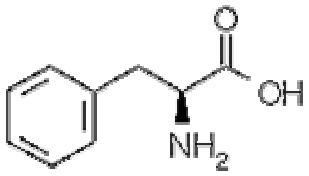
leucine* (**Leu, L**)



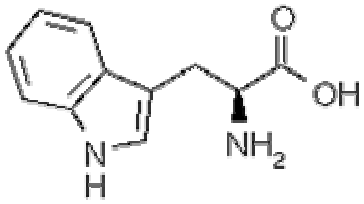
isoleucine* (**Ile, I**)



methionine* (**Met, M**)

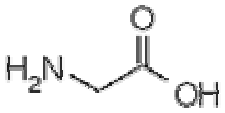


phenylalanine* (**Phe, F**)

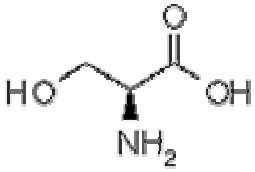


tryptophan* (**Trp, W**)

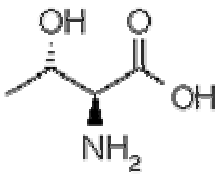
Polar, uncharged (hydrophilic)



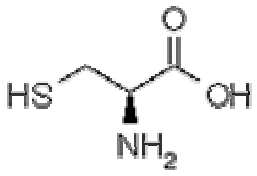
glycine (**Gly, G**)



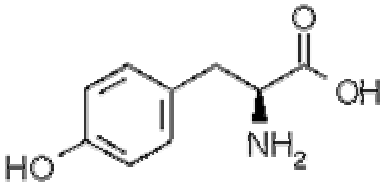
serine (**Ser, S**)



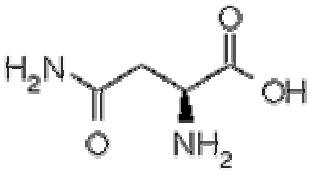
threonine* (**Thr, T**)



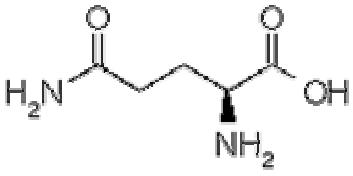
cysteine (**Cys, C**)



tyrosine (**Tyr, Y**)

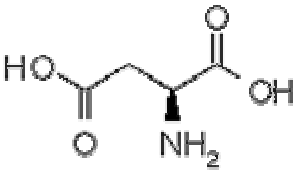


asparagine (**Asn, N**)

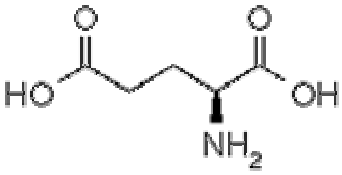


glutamine (**Gln, Q**)

Acidic, negatively charged (hydrophilic)

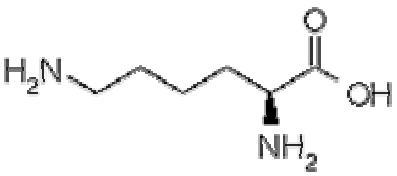


aspartate (**Asp, D**)

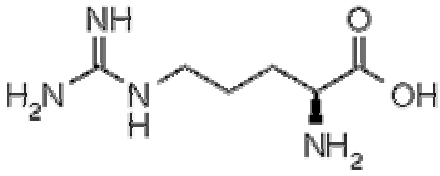


glutamate (**Glu, E**)

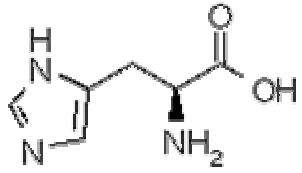
Basic, positively charged (hydrophilic)



lysine* (**Lys, K**)



arginine (**Arg, R**)



histidine* (**His, H**)

*dietary essential amino acids

SUMMARY

SAMENVATTING

Summary

Hypertension or high blood pressure is a major risk factor for the development of cardiovascular diseases, which are the leading causes of morbidity and mortality in western society. Recently, some functional foods have received considerable attention for their effectiveness in both the prevention and the treatment of hypertension. This is among others due to the presence of food derived bioactive peptides with potential antihypertensive properties. Compared to antihypertensive drugs, these peptides, as part of a (functional) food or as nutraceutical, have no side effects and are less expensive. Moreover, as a more natural product, they appeal to the consumer.

Angiotensin I converting enzyme (ACE) inhibitory peptides exert an antihypertensive effect by inhibition of the angiotensin I converting enzyme in the cardiovascular system, hence preventing the formation of the potent vasoconstrictor angiotensin II and the degradation of the vasodilator bradykinin. In order to reduce the blood pressure after oral administration, ACE inhibitory peptides have to reach the bloodstream in an active form. Therefore, stability against human proteases and intestinal transport of active peptides is required. In the current field of ACE inhibitory peptide research, the maintenance of the activity in the oral delivery route is often not taken into account. ACE inhibitory peptides have mainly been isolated from milk proteins and have not yet been studied from pea protein.

The aim of this work was to investigate the release of ACE inhibitory activity from pea and whey protein. Moreover, whey protein, from which certain bioactive peptides already have been characterised, was compared to the unknown pea protein. Emphasis was placed on the *in vitro* study of the activity of ACE inhibitory peptides after oral administration in the human body, from the gastrointestinal tract to their site of action, the cardiovascular system.

A diagnostic assay to measure ACE activity was transformed into an ACE inhibition assay and subsequently optimised to obtain a more sensitive and less expensive test. This spectrophotometric method, where ACE inhibition was measured with the substrate FAPGG and rabbit lung acetone extract as ACE source, was validated by the antihypertensive drugs captopril, enalapril and its active derivative enalaprilat, and by the lactokinin Ala-Leu-Pro-Met-His-Ile-Arg. In addition, it indicated the release of ACE inhibitory activity after digestion of pea and whey protein. A more delicate and standardised assay was obtained by applying

pure ACE from porcine kidney as ACE source. The ACE inhibitory activity of captopril and Ala-Leu-Pro-Met-His-Ile-Arg was also verified in this assay. The ACE inhibition assay presented a relatively simple and reliable tool to screen for ACE inhibitory peptides from food proteins.

The activity of the potent ACE inhibitory peptide Ala-Leu-Pro-Met-His-Ile-Arg, originally derived from a tryptic digest of β-lactoglobulin, was investigated *in vitro* in the oral delivery route. Protein digestion in the gastrointestinal tract was simulated by digestion of a 4% (w/v) protein solution with porcine pepsin at pH 2 for 2 h and subsequent digestion with bovine trypsin and bovine α-chymotrypsin at pH 6.5 for 2.5 h at 37°C; all enzymes in an enzyme over substrate ratio of 1/250 (w/w). During *in vitro* gastrointestinal digestion, the heptapeptide was partially degraded by α-chymotrypsin, likely to Ala-Leu-Pro-Met and His-Ile-Arg, the latter also possessing ACE inhibitory activity. Nevertheless, after hydrolysis by stomach and pancreatic proteases, half of the initial peptide concentration and 70% of the initial ACE inhibitory activity could still be recovered. The lactokinin was rapidly broken down by rat intestinal tissue peptidases, while almost no degradation was observed after incubation with Caco-2 homogenates. The intestinal transport of 1 mM of the lactokinin was investigated in a Caco-2 Bbe cell monolayer mounted in an Ussing chamber. After 10 min of incubation at 37°C, substantial ACE inhibitory activity was found in the serosal compartment after threefold concentration of the samples. Concomitantly, MALDI-TOF spectrometry detected the heptapeptide in the serosal compartment. Under the observed experimental conditions, the ACE inhibitory peptide Ala-Leu-Pro-Met-His-Ile-Arg was transported intact through the Caco-2 Bbe cell monolayer, although at low concentrations. It seems that the degradation by intestinal peptidases constitutes the major barrier in the oral delivery route of this lactokinin.

Fermentation by different lactic acid bacteria was studied as a means to release ACE inhibitory activity from pea and whey protein. Fermentation of pea protein by *Lactobacillus helveticus* yielded the highest ACE inhibitory activity and was therefore selected for subsequent experimentation. Next, pea and whey protein were fermented by *Lactobacillus helveticus* and the yeast *Saccharomyces cerevisiae* in monoculture and in combination at 28 and 37°C. Fermentation was always followed by *in vitro* gastrointestinal digestion and the digests of non-fermented protein solutions served as controls. After fermentation, the ACE inhibitory activity (%) of a 2.73 mg/ml sample increased by 18 to 30% for all treatments, except for the fermentations of whey protein by *Saccharomyces cerevisiae* at 28°C, where no significant change was observed. After digestion, however, both fermented and non-fermented samples reached maximal ACE inhibitory activity (%). The degree of proteolysis showed only a minor increase after fermentation and augmented sharply after digestion. The whey (fermented) digests tended to have lower 50% inhibitory concentrations (IC_{50}) (0.148-

0.072 mg/ml), hence higher ACE inhibitory activity, than the pea (fermented) digests (0.183-0.093 mg/ml). The non-fermented digested samples were at least as ACE inhibitory active as the fermented ones. The non-fermented whey protein digest showed the lowest IC_{50} value and hence the highest ACE inhibitory activity of all. For pea protein, the non-fermented sample had the one but lowest IC_{50} value. These results suggest that *in vitro* gastrointestinal digestion was the predominant factor controlling the formation of ACE inhibitory activity, hence indicating its importance in the bioavailability of ACE inhibitory peptides.

Therefore, the formation of ACE inhibitory activity during *in vitro* gastrointestinal digestion of pea and whey protein was investigated more profoundly.

Firstly, the conditions during *in vitro* gastrointestinal digestion of pea and whey protein leading to maximal release of ACE inhibitory activity were characterised. In batch experiments, three *in vitro* gastrointestinal digestions varying in pH and incubation time in the stomach and the small intestine phase, were compared for pea and whey protein. The digestion simulating the physiological conditions of protein hydrolysis in the human body sufficed to achieve the highest ACE inhibitory activity, with IC_{50} values of 0.076 mg/ml for pea and 0.048 mg/ml for whey protein. The degree of proteolysis did not correlate with the ACE inhibitory activity and pea protein was more susceptible to hydrolysis by gastrointestinal proteases than whey protein. In a semi-continuous reactor model of gastrointestinal digestion (pre-SHIME), response surface methodology was used to investigate the influence of temperature, incubation time in the stomach phase and incubation time in the small intestine phase on the ACE inhibitory activity and the degree of proteolysis. For the pea protein, a central composite design could constitute a linear model for the degree of proteolysis and a quadratic model for the ACE inhibitory activity, expressed as log IC_{50}. Within the model, maximal degree of proteolysis was observed at the highest temperature and the longest incubation time in the small intestine phase, while maximal ACE inhibitory activity was obtained at the longest incubation times in the stomach and the small intestine phase. For the whey protein, no significant model for both responses could be designed. Yet, the overall results demonstrate that the ACE inhibitory activity of pea and whey protein hydrolysates can be controlled by the conditions of the *in vitro* gastrointestinal digestion.

Next, the evolution of ACE inhibitory activity, degree of proteolysis and protein degradation was investigated during *in vitro* physiological digestion. A 2.73 mg/ml pea digest showed maximal ACE inhibitory activity (%) already in the early stomach phase and its IC_{50} further decreased in the small intestine phase. For whey digest, however, the level of 100% ACE inhibitory activity was only attained in the small intestine phase. Subsequent supplementation of a rat intestinal acetone powder, which simulated the digestion by brush border enzymes, resulted in an increase in IC_{50} value for both proteins. This decrease in ACE

inhibitory activity was less pronounced for pea compared to whey. Yet, a substantial amount of peptides were still ACE inhibitory active after digestion of pea and whey protein by gastrointestinal and brush border enzymes and the final IC_{50} values were 0.093 mg/ml for pea and 0.128 mg/ml for whey. The degree of proteolysis presented a different evolution and augmented more for pea than for whey in both the stomach and small intestine phase, while the brush border peptidases caused a smaller increase in the degree of proteolysis of pea compared to whey. SDS-PAGE showed that the major part of the proteins in pea was already broken down in the stomach phase. The major whey protein β-lactoglobulin was only degraded from the small intestine phase onwards. The presence of known ACE inhibitory peptides in the pea and whey protein sequences was studied using a dedicated computer program. This program makes use of a database of about 500 reported ACE inhibitory sequences and their IC_{50} values, and produces a theoretical score, weighted on the total number of amino acids, for the potential of ACE inhibitory peptides in a given source protein. For pea, vicilin (VCLC_PEA) and albumin PA2 (PA2_PEA) obtained scores as high as 10 on ACE inhibitory activity, compared to 16 for β-casein (CASB_BOVIN) taken as a reference protein. β-lactoglobulin (LACB_BOVIN) exceeded all other proteins with a score of 26. To our knowledge, this is the first described large ACE inhibitory peptide database that attributes scores for the potential of ACE inhibitory activity to protein sequences. *In silico* digestion of these proteins by pepsin, trypsin and α-chymotrypsin directly released two ACE inhibitory peptides from PA2, one from vicilin and one from β-lactoglobulin with an IC_{50} lower than 100 µM. Furthermore, several less active ACE inhibitory peptides and precursor peptides, which may form ACE inhibitory peptides upon further digestion by brush border, enterocyte or plasma peptidases, were released. The high ACE inhibitory peptide score of β-lactoglobulin and the fact that this protein resists digestion by pepsin are consistent with the substantial decrease in IC_{50} for the whey digest during the small intestine phase digestion. The database and *in silico* digestion facilitate the efficient release of ACE inhibitory peptides and the study of proteins on a large scale.

The ACE inhibitory activity of pea and whey hydrolysate, obtained by *in vitro* gastrointestinal digestion, with IC_{50} values of 0.070 mg P/ml and 0.041 mg P/ml, was further increased upon purification by ultrafiltration-centrifugation and subsequent fractionation of the permeate by RP-HPLC. The most active RP-HPLC fractions eluted at 24-28% acetonitrile and therefore contained short, hydrophobic peptides, which is in accordance with the structure-activity relationship of ACE inhibitory peptides. The IC_{50} values of these fractions amounted to 0.016 mg P/ml for pea and 0.003 mg P/ml for whey. In this way, the ACE inhibitory activity of the pea digest was more than 4 times enriched, while that of the whey digest was more than 13 times augmented. This may suggest that in whey hydrolysate very

potent ACE inhibitory peptides were present next to low active peptides, while in pea digest all peptides had similar ACE inhibitory activity.

The intestinal transport of active compounds in the digests and permeates was investigated in Caco-2 cell monolayers. All samples retained relatively high ACE inhibitory activity after 2 h incubation in the presence of Caco-2 homogenates. Addition of 9 mg P/ml digests and 10 mg P/ml permeates in the apical compartments of the Caco-2 cell monolayers resulted in the detection of no or only little ACE inhibitory activity in the basolateral compartments after 1 h transport experiment, even after concentration of the sample. Some ACE inhibitory activity was observed in the transport experiments after addition of 45 mg P/ml digests and 50 mg P/ml permeates in the apical compartments, but these were associated with compromised cell monolayer integrity as indicated by decreased transepithelial electrical resistance and increased sodium fluorescein fluxes. Hence, not the degradation by intestinal peptidases, but the uptake by the cell monolayer seemed to be the decisive factor with respect to the transport of active compounds in the ACE inhibitory digests and permeates. As the Caco-2 model is tighter than intestinal mammalian tissue, transport of these peptides in substantial quantities might still take place *in vivo*.

After intravenous administration of a dose of 50 mg P/kg BW in spontaneously hypertensive rats, pea permeate exerted a transient, but strong antihypertensive effect of 44.4 mmHg. Whey permeate exerted no effect at a dose of 50 mg P/kg BW. Further research into potential positive effects in the prevention and treatment of hypertension, for example after daily administration over a longer period of time or after addition of a single higher dose, is necessary.

In conclusion, this work presented a profound investigation on the release of ACE inhibitory activity from pea and whey protein and the maintenance of this activity in the oral delivery route. For the first time, the formation of high ACE inhibitory activity from pea protein is described. This knowledge can be applied in functional foods for the prevention and treatment of hypertension.

Samenvatting

Hypertensie of hoge bloeddruk is een belangrijke risicofactor voor de ontwikkeling van cardiovasculaire ziekten, die in de Westerse wereld de voornaamste doodsoorzaak zijn. Recent hebben een aantal functionele voedingsmiddelen belangstelling gewekt omwille van hun effectiviteit in de preventie en behandeling van hypertensie. Dit is onder meer te wijten aan de aanwezigheid van uit voedingseiwit afkomstige bioactieve peptiden met potentieel antihypertensieve eigenschappen. Vergeleken met de antihypertensieve geneesmiddelen hebben deze peptiden, aanwezig in een (functioneel) voedingsmiddel of als nutraceutical, het voordeel dat ze geen ongewenste neveneffecten teweegbrengen en minder duur zijn. Bovendien spreken deze meer natuurlijke producten de consument aan.

Angiotensin I converting enzyme (ACE) - inhibitorische peptiden oefenen een antihypertensief effect uit door inhibitie van het angiotensin I converting enzyme in het cardiovasculair systeem, wat de vorming van de vasoconstrictor angiotensine II en de afbraak van de vasodilator bradykinine bewerkstelligt. Opdat deze ACE-inhibitorische peptiden na orale inname een antihypertensief effect zouden uitoefenen, is het noodzakelijk dat ze de bloedbaan in een actieve vorm bereiken. Daarvoor is enige stabiliteit ten opzichte van proteasen en intestinaal transport van actieve peptiden in het menselijk lichaam vereist. In het huidige, wetenschappelijke domein van ACE-inhibitorische peptiden wordt het behoud van de activiteit na orale inname in het menselijk lichaam vaak niet in rekening gebracht. ACE-inhibitorische peptiden zijn hoofdzakelijk geïsoleerd uit melkeiwitten en erwteneiwit is in dit verband nog niet bestudeerd.

De voornaamste doelstelling van dit onderzoek was dan ook kennis te verwerven over de vrijstelling van ACE-inhibitorische peptiden uit erwten- en wei-eiwit. Bovendien werd wei-eiwit, waarvan reeds een aantal bioactieve peptiden gekarakteriseerd werden, vergeleken met het ongekende erwteneiwit. De nadruk werd hierbij gelegd op het nagaan *in vitro* van de activiteit van ACE-inhibitorische peptiden na orale inname in het menselijk lichaam, vanaf het gastro-intestinaal kanaal tot aan het cardiovasculair systeem, waar ze hun activiteit uitoefenen.

Een diagnostische kit voor het meten van ACE-activiteit werd omgevormd tot een ACE-inhibitietest en vervolgens geoptimaliseerd tot een gevoeligere en goedkopere test. Deze

spectrofotometrische methode, waarbij de ACE-inhibitie gemeten werd met FAPGG als substraat en konijnenlong-acetonextract als bron van ACE, werd gevalideerd door de antihypertensieve geneesmiddelen captopril, enalapril en de actieve verbinding enalaprilat, en door het lactokinine Ala-Leu-Pro-Met-His-Ile-Arg. Deze test toonde bovendien de vrijstelling van ACE-inhibitorische activiteit aan na vertering van erwten- en wei-eiwit. Een gevoeligere en gestandaardiseerde ACE-inhibitietest werd bekomen door het aanwenden van zuiver ACE afkomstig uit varkensnieren. De ACE-inhibitorische activiteit van captopril en Ala-Leu-Pro-Met-His-Ile-Arg werd eveneens met deze test bepaald. De ACE-inhibitietest bleek in staat om op een relatief eenvoudige en betrouwbare manier te screenen naar ACE-inhibitorische peptiden uit voedingseiwitten.

De activiteit van het krachtige ACE-inhibitorisch peptide Ala-Leu-Pro-Met-His-Ile-Arg, oorspronkelijk afkomstig van een trypsine-hydrolysaat van β-lactoglobuline, werd *in vitro* onderzocht in de orale toeleveringsweg. De eiwitvertering in het gastro-intestinaal kanaal werd nagebootst door vertering van een 4% (w/v) eiwitoplossing met varkenspepsine bij pH 2 gedurende 2 h en vervolgens met rundertrypsine en runder-α-chymotrypsine bij pH 6.5 gedurende 2.5 h bij 37°C; alle enzymen in een enzym-over-substraatverhouding van 1/250 (w/w). Tijdens deze *in vitro* gastro-intestinale vertering werd het heptapeptide gedeeltelijk afgebroken door α-chymotrypsine, waarschijnlijk tot Ala-Leu-Pro-Met en His-Ile-Arg, wat ook ACE-inhibitorische activiteit heeft. Toch bleef er na de vertering met maag- en pancreasproteasen nog de helft van de initiële peptideconcentratie en 70% van de initiële ACE-inhibitorische activiteit over. Peptidasen uit rat intestinaal weefsel braken het lactokinine zeer snel af, terwijl er in de aanwezigheid van Caco-2 homogenaten nauwelijks een afbraak werd waargenomen. Het intestinaal transport van 1 mM van het lactokinine werd nagegaan in een Caco-2 Bbe celmonolayer gemonteerd in een Ussing kamer. Na 10 min incubatie bij 37°C werd er na driemaal opconcentratie aanzienlijke ACE-inhibitorische activiteit waargenomen in de stalen van het serosale compartiment. Tevens werd het heptapeptide door MALDI-TOF spectrometrie in het serosale compartiment gedetecteerd. Onder de gegeven experimentele omstandigheden werd het ACE-inhibitorisch peptide Ala-Leu-Pro-Met-His-Ile-Arg intact getransporteerd doorheen de Caco-2 Bbe celmonolayer, weliswaar in een lage concentratie. Blijkbaar vormt de degradatie door intestinale peptidasen het voornaamste knelpunt in de orale toeleveringsweg van dit lactokinine.

Fermentatie door verschillende melkzuurbacteriën werd onderzocht als procedure om ACE-inhibitorische activiteit vrij te stellen uit erwten- en wei-eiwit. Fermentatie van erwteneiwit met *Lactobacillus helveticus* bleek het meest effectief en werd daarom geselecteerd voor verdere proeven. Daarbij werden erwten- en wei-eiwit gefermenteerd door *Lactobacillus helveticus* en de gist *Saccharomyces cerevisiae* in monocultuur en in

combinatie bij 28 en 37°C. Na fermentatie werd steeds een *in vitro* gastro-intestinale vertering uitgevoerd en verteerde, niet-gefermenteerde eiwitoplossingen dienden als controle. De ACE-inhibitorische activiteit (%) van een 2.73 mg/ml staal nam na fermentatie toe met 18 tot 30% in alle behandelingen, met uitzondering van wei-eiwit gefermenteerd door *Saccharomyces cerevisiae* bij 28°C, waar geen significante verandering werd vastgesteld. Na vertering bereikten echter zowel gefermenteerde als niet-gefermenteerde stalen 100% ACE-inhibitorische activiteit. De proteolysegraad vertoonde een beperkte toename na fermentatie en steeg aanzienlijk na vertering. De (gefermenteerde) wei-eiwithydrolysaten leken te beschikken over een lagere 50% inhibitorische concentratie (IC_{50}) (0.148-0.072 mg/ml) en dus een hogere ACE-inhibitorische activiteit dan de (gefermenteerde) erwteneiwithydrolysaten (0.183-0.093 mg/ml). Hierbij vertoonden de niet-gefermenteerde, verteerde stalen minstens evenveel ACE-inhibitorische activiteit als de gefermenteerde, verteerde stalen. Het niet-gefermenteerde wei-eiwithydrolysaat bezat de laagste IC_{50}-waarde en dus de hoogste ACE-inhibitorische activiteit. Bij het erwteneiwit had het niet-gefermenteerde staal de op één na laagste IC_{50}-waarde. Deze resultaten suggereren dat hoofdzakelijk de *in vitro* gastro-intestinale vertering de vorming van ACE-inhibitorische activiteit bepaalt, wat wijst op het belang ervan in de bio-beschikbaarheid van ACE-inhibitorische peptiden.

Bijgevolg werd de vrijstelling van ACE-inhibitorische activiteit tijdens de *in vitro* gastro-intestinale vertering van erwten- en wei-eiwit meer in detail onderzocht.

Eerst werden de omstandigheden nodig voor een maximale vrijstelling van ACE-inhibitorische activiteit tijdens de *in vitro* gastro-intestinale vertering van erwten- en wei-eiwit gekarakteriseerd. In batch experimenten werden drie *in vitro* gastro-intestinale verteringen, verschillend in pH en incubatieduur in de maag en de dunne darm fase, vergeleken voor erwten- en wei-eiwit. De vertering die de fysiologische omstandigheden van de eiwitvertering in het menselijk lichaam nabootste, had de hoogste ACE-inhibitorische activiteit, met IC_{50} waarden van 0.076 mg/ml voor erwten- en 0.048 mg/ml voor wei-eiwit. Er was geen verband tussen de proteolysegraad en de ACE-inhibitorische activiteit en erwten-eiwit was meer onderhevig aan hydrolyse door gastro-intestinale proteasen dan wei-eiwit. In een semi-continu reactormodel van de gastro-intestinale vertering (pre-SHIME), werd door middel van responsoppervlak-methodologie de invloed van temperatuur, incubatieduur in de maagfase en incubatieduur in de dunne darmfase nagegaan op de ACE-inhibitorische activiteit en de proteolysegraad. Voor het erwteneiwit werd met behulp van een centraal samengesteld design een lineair model opgesteld voor de proteolysegraad en een kwadratisch model voor de ACE-inhibitorische activiteit, uitgedrukt als log IC_{50}. Binnen de grenzen van het model, werd een maximale proteolysegraad waargenomen bij de hoogste temperatuur en de langste

incubatieduur in de dunne darmfase, terwijl maximale ACE-inhibitorische activiteit bereikt werd bij de langste incubatieduren in de maag en de dunne darmfase. Voor beide responsen van het wei-eiwit kon geen significant model worden opgesteld. Algemeen tonen deze resultaten aan dat de ACE-inhibitorische activiteit van erwten- en wei-eiwithydrolysaten kan bepaald worden door de condities van de *in vitro* gastro-intestinale vertering.

Vervolgens werd het verloop van de ACE-inhibitorische activiteit, de proteolysegraad en de eiwitafbraak tijdens de *in vitro* fysiologische vertering bestudeerd. Een 2.73 mg/ml erwteneiwithydrolysaat vertoonde reeds maximale ACE-inhibitorische activiteit (%) in de vroege maagfase en zijn IC_{50} nam verder af tijdens de dunne darmfase. Voor wei-eiwithydrolysaat werd het niveau van 100% ACE-inhibitorische activiteit pas bereikt in de dunne darmfase. Toevoeging van een rat intestinaal acetonpoeder, wat de vertering door brushborderenzymen simuleerde, had een stijging in IC_{50} waarde voor beide ewithydrolysaten tot gevolg. Deze afname in ACE-inhibitorische activiteit was minder uitgesproken bij erwt dan bij wei. Toch was er nog een aanzienlijke hoeveelheid ACE-inhibitorische activiteit aanwezig na vertering van erwten- en wei-eiwit met gastro-intestinale en brushborderenzymen en de uiteindelijke IC_{50} waarden bedroegen 0.093 mg/ml voor erwt en 0.128 mg/ml voor wei. De proteolysegraad kende een ander verloop en steeg meer voor erwt dan voor wei zowel in de maag als in de dunne darmfase, terwijl de brushborderpeptidasen een kleinere toename in de proteolysegraad bij erwt veroorzaakten dan bij wei. SDS-PAGE toonde dat het merendeel van de eiwitten in erwt reeds afgebroken werd tijdens de maagfase-vertering. Het voornaamste wei-eiwit, β-lactoglobuline, werd pas afgebroken vanaf de dunne darmfase. De aanwezigheid van gekende ACE-inhibitorische peptiden in de verschillende eiwitsequenties in erwt en wei werd bestudeerd door middel van een specifiek computerprogramma. Dit programma maakt gebruik van een database van ongeveer 500 gekende ACE-inhibitorische peptidesequenties en hun IC_{50} waarden, en berekent een theoretische score, gestandaardiseerd op de totale hoeveelheid aminozuren, voor het potentieel aan aanwezige ACE-inhibitorische peptiden in een gegeven eiwit. Bij erwt scoorden viciline (VCLC_PEA) en albumine PA2 (PA2_PEA) het hoogst op ACE-inhibitorische activiteit met een waarde 10, in vergelijking met 16 voor β-caseïne (CASB_BOVIN) dat als referentie-eiwit werd genomen. β-lactoglobuline (LACB_BOVIN) stak boven alle andere eiwitten uit met een score van 26. Voor zover wij weten is dit de eerste beschrijving van een grote ACE-inhibitorische peptidendatabase die scores van potentieel aan ACE-inhibitorische activiteit toekent aan eiwitsequenties. *In silico* vertering van deze eiwitten met pepsine, trypsine and α-chymotrypsine stelde rechtstreeks twee ACE-inhibitorische peptiden vrij uit PA2, één uit viciline en één uit β-lactoglobuline met een IC_{50} lager dan 100 µM. Bovendien werden er een aantal minder actieve ACE-inhibitorische

peptiden en precursorpeptiden, die ACE-inhibitorische peptiden kunnen vormen na verdere vertering met brushborder-, enterocyt- of plasmapeptidasen, vrijgesteld. De hoge ACE-inhibitorische peptide score van β-lactoglobuline en het feit dat dit eiwit moeilijk wordt afgebroken door pepsine, stemden overeen met de sterke afname in IC_{50} voor het wei-eiwit tijdens de dunne darmfase. De databank en *in silico* vertering maken een doeltreffendere vrijstelling van ACE-inhibitorische peptiden en studie van eiwitten op grote schaal mogelijk.

De ACE-inhibitorische activiteit van het erwten- en wei-eiwithydrolysaat, verkregen door gastro-intestinale vertering, met IC_{50} waarden van 0.070 mg P/ml en 0.041 mg P/ml, werd verder verhoogd door opzuivering met ultrafiltratie-centrifugatie en RP-HPLC. De meest actieve RP-HPLC fracties werden opgevangen bij 24-28% acetonitrile en bevatten bijgevolg korte, hydrofobe peptiden, wat in overeenstemming is met de structuur-activiteitsrelatie van ACE-inhibitorische peptiden. De IC_{50} waarden van deze fracties waren 0.016 mg P/ml voor erwt en 0.003 mg P/ml voor wei. Zo werd de ACE-inhibitorische activiteit van het erwteneiwithydrolysaat meer dan viermaal verrijkt, terwijl deze van wei-eiwithydrolysaat meer dan dertienmaal toenam. Dit suggereert dat in wei-eiwithydrolysaat zeer krachtige ACE-inhibitorische peptiden aanwezig waren naast peptiden met lage activiteit, terwijl in erwteneiwithydrolysaat alle peptiden gelijkaardige ACE-inhibitorische activiteit vertoonden.

Het intestinaal transport van actieve componenten in deze hydrolysaten en permeaten werd onderzocht in het Caco-2 celmonolayer model. Alle stalen vertoonden nog hoge ACE-inhibitorische activiteit na 2 h incubatie in de aanwezigheid van Caco-2 homogenaten. Toevoeging van 9 mg P/ml hydrolysaten en 10 mg P/ml permeaten aan de apicale compartimenten van de Caco-2 celmonolayers resulteerde in de detectie van geen of zeer weinig ACE-inhibitorische activiteit in de basolaterale compartimenten na 1 h transportexperiment, zelfs na opconcentratie. Lage ACE-inhibitorische activiteit werd wel waargenomen in de transportexperimenten na toevoeging van 45 mg P/ml hydrolysaten en 50 mg P/ml permeaten in de apicale compartimenten, maar deze resulteerden in beschadiging van de celmonolayer, wat zich uitte in verlaagde transepitheliale elektrische weerstanden en verhoogde natriumfluoresceïne fluxen. Vandaar leek niet zozeer de degradatie door intestinale peptidasen, maar wel de opname door de celmonolayer het transport van ACE-inhibitorische hydrolysaten en permeaten te bepalen. Aangezien de cellen in het Caco-2 model dichter bij elkaar aansluiten dan in intestinaal zoogdierenweefsel, is het toch mogelijk dat transport van deze peptiden *in vivo* in voldoende hoeveelheden plaatsvindt.

Na intraveneuze toediening van een dosis van 50 mg P/kg BW in spontaan hypertensieve ratten, oefende erwtenpermeaat een tijdelijk, maar sterk antihypertensief effect uit van 44.4 mmHg. Weipermeaat oefende geen effect uit bij een dosis van 50 mg P/kg BW. Verder

onderzoek is vereist naar potentiële positieve effecten in de preventie en de behandeling van hypertensie, na dagelijkse toediening over een langere periode of na toevoeging van een enkele hogere dosis.

Samenvattend bestond dit werk uit een diepgaand onderzoek naar de vrijstelling van ACE-inhibitorische activiteit uit erwten- en wei-eiwit en het behoud van deze activiteit in de orale toeleveringsweg. Voor het eerst wordt de vorming van hoge ACE-inhibitorische activiteit uit erwteneiwit beschreven. Deze kennis kan toegepast worden in functionele voeding voor de preventie en behandeling van hypertensie.

CHAPTER 1

LITERATURE REVIEW

Chapter 1

Literature review

1. NUTRITION FOR OPTIMAL HEALTH

1.1. Nutrition in relation to health

Nowadays, it is well-recognised that nutrition has a major impact on human health. The way of looking at nutrition and nutrition science has undergone major changes the last and current century, which are associated at least in the Western world with the transformation from food scarcity to food abundance for the preponderance of the population and an increase in average lifespan that stresses the importance of quality of life.

In the first part of the twentieth century, nutrition science made unexpected discoveries that completed the understanding of essential nutrients and their concomitant deficiency disorders. This led to the recommended dietary allowances for nutrients, which are still in use for dietary advice and food consumption surveys today. The diet was attributed merely a passive role in that the presence in food products of nutrients in sufficient amounts was considered a condition for attaining or maintaining a good health status. Therefore, a strong focus was placed on the importance of variety in the diet to fulfil the need for adequate nutrition. From the second part of the twentieth century on, human requirements of nutrients were defined even more explicitly. Food technology made much progress and this resulted in industrially prepared products with defined nutrient composition. Gradually the awareness grew that good nutrition is not just a matter of a sufficient supply of nutrients. Epidemiologic evidence linked the prevalence of diseases of civilisation or affluence such as cardiovascular disease, obesity, hypertension, type 2 diabetes and certain types of cancer to specific dietary factors. Diet is not the sole cause, since these diseases are due to interactions of multiple factors, including heredity, stress, environmental pollution, smoking and lack of exercise. Nonetheless, diet is a factor that is readily amenable to change. A transition in nutrition science took place in which nutrition played a more active role focussing on health promotion. From ca 1970 on, the search for optimal nutrition began. This resulted in the dissemination of dietary guidelines by health authorities in many Western countries. Moreover, a new concept in nutrition science, that of functional foods, was introduced to make a further contribution to disease prevention (Bender, 1997; Diplock *et al.*, 1999;

Schaafsma, 1997). In this respect, a number of bioactive compounds have been characterised in natural, modified and novel foods (Table 1).

Table 1. Examples of candidate functional food components for modulation of certain target functions (Diplock *et al.*, 1999).

Target functions	Candidate food components
Skeletal development	Calcium, vitamin D, vitamin C
Immune function	Vitamin A, vitamin D, antioxidant vitamins, n-3 and n-6 PUFA, trace elements, arginine, nucleotides and nucleosides, probiotics
Defence of DNA, PUFA and (lipo)proteins against reactive oxidative species	Vitamin E, vitamin C, carotenoids, polyphenols including flavonoids, selenium
Control of hypertension	Total energy (↓), sodium chloride (↓), n-3 PUFA from fish
Optimal intestinal function and stool formation	Non-digestible carbohydrates, probiotics, prebiotics, synbiotics
Cognitive performance	Glucose, caffeine, B vitamins, choline

1.2. Functional foods

1.2.1. Definition

No universally accepted definition for functional foods exists. A rather extensive description of the term is given in the Consensus Document of the Functional Food Science in Europe (FUFOSE) supported by the European Union and coordinated by the International Life Science Institute (ILSI) Europe (Diplock *et al.*, 1999). A food can be regarded as 'functional' if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. Functional foods must remain foods and they must demonstrate their effects in amounts that can normally be expected to be consumed in the diet: they are no pills or capsules, but part of a normal food pattern. A functional food can be a natural or modified food and it might be functional for all members of the population or just for a particular group.

The suggestion that certain foods or their components might offer some unique health benefits has blurred the distinction between foods and drugs. Therefore, it is suggested to deal with functional foods and their relationship to health at a level that ascribes to

prevention, a reduced risk of disease or an improved biological function. Moreover, the consumption of food is usually regarded as safe, while for drugs a benefit-risk ratio applies. However, scientific studies should not overlook the relative benefits or risks that might accrue from the increased ingestion of functional foods (Clydesdale, 1997; Milner, 2000).

Functional foods are often confused with novel foods and nutraceuticals although these terms refer to different food categories. Although functional foods may be novel, this novelty is not a strict necessity. Novel foods and novel food ingredients are food products or food ingredients not used to date in significant quantities, the method of production or the composition of which is different as marketed thus far (ILSI, 1996). Nutraceuticals are products produced from food, but sold in pills, powders and other medicinal forms not generally associated with food, and which have shown a physiological benefit or a reduced risk of chronic disease (Clydesdale, 1997).

There are a number of distinct types of claims concerning the association between nutrition and health, and the definition of them is not the same in all countries.

1.2.2. Japan

The concept of functional foods was introduced in Japan in the late 1980s. Public awareness grew that lifestyle related diseases could be prevented through improved dietary practice in the daily life. In 1984, the first national project on functional foods commenced under the sponsorship of the Ministry of Education, Science and Culture, which was entitled: 'Systematic analysis and development of food functions'. One of the functions was newly defined as the body-modulating function of non-nutrients that is directly or indirectly related to disease prevention. In 1991, the Ministry of Health and Welfare established the world's first policy to legally permit the commercialisation of some functional foods in terms of 'Foods for Specified Health Uses (FOSHU)'. Upon satisfactory submission of comprehensive data documenting the scientific evidence of the food product, this policy is able to approve a health claim and grant permission to use a label, indicating to the consumer that the food product is identified as FOSHU (Arai *et al.*, 2001; Sanders and Huis in 't Veld, 1999). At the end of the year 2000, health claims for 192 food products as FOSHU had been permitted and most of them contained either oligosaccharides or lactic acid bacteria for promoting intestinal health (Arai *et al.*, 2001; Diplock *et al.*, 1999).

The functional food science in Japan has been progressing strongly to occupy a central position in modern bioscience. It is especially characterised by the strength in a product-driven way of study, which may be influenced by local, traditional or cultural characteristics (Arai *et al.*, 2002).

1.2.3. United States

Three US legislative acts have likely had a significant effect on the promotion of the concept of functional foods. Firstly, the Nutrition Labelling and Education Act (NLEA), passed in 1990, allowed foods to bear a health claim under the condition that the claim was approved by the Food and Drug Administration (FDA). A health claim is a statement that characterises a relationship between any food substance in a diet and a disease or health-related condition, e.g. ‘protects against coronary heart disease’ (Sanders and Huis in 't Veld, 1999). To date, 14 such claims have been evaluated and approved by the FDA (Sanders, 2002). Secondly, the Dietary Supplement Health and Education Act of 1994 defined dietary supplements and created a mechanism for dealing with safety issues, regulation of health claims and labelling. Thirdly, the FDA Modernisation Act of 1997 even allowed health claims that are not preauthorised by FDA, if they are based on authoritative statements of a Federal Scientific Body such as the National Academy of Sciences or the National Institutes of Health and a notification to the FDA is made (Milner, 2000).

A health claim differs from a nutrient content claim, which either explicitly or by implication, characterises the level of any nutrient required to be listed on the nutrition label, like ‘reduced cholesterol’. These descriptors must be used in accordance with FDA and US Department of Agriculture definitions. In addition to this, structure/function claims are allowable on foods and dietary supplements; they describe the effect of a food on a structure or function of the body e.g. ‘helps maintain cardiovascular health’, for which no prior approval by the FDA is required, although notification is necessary (Clydesdale, 1997; Sanders and Huis in 't Veld, 1999). The road to health claim approval is a long and costly one, not undertaken by food companies lightly. Nor in some cases is a disease claim considered to be a marketing advantage. An alternative is the use of the structure/function claim (Sanders, 2002).

1.2.4. Europe

In the European Union, there is no harmonised legislation on functional nutritional or health claims, which means that they are dealt with at the national level. The Codex Alimentarius from the World Health Organisation (WHO) has recently classified and defined four different forms of claims (Codex Alimentarius, 1997). In the European Consensus Document two further types of claims are proposed in the context of functional foods: enhanced function claims and reduction of disease-risk claims (Table 2).

Table 2. Different food claims as classified by the Codex Alimentarius (type 1 to 4) and the European Consensus Document on Functional Foods (type A and B) (Codex Alimentarius, 1997; Diplock *et al.*, 1999).

	Definition	Example(s)
1	Claims related to dietary guidelines or healthy diets officially recognised by the appropriate national authority	The advice of … is to choose a diet high in fibre, low in saturated fats, … .
2	*Nutrient content claims*	Source of calcium, high in fibre, low in fat, … .
3	*Comparative claims* between the nutrient level of two or more foods	Reduced, less than, increased, more than, … .
4	*Nutrient function claims* with a reference to the physiological role of a nutrient in its relationship to growth, development and normal functions of the body	Calcium might help the development of strong bones and teeth.
A	*Enhanced function claims* that concern specific beneficial effects of (non-)nutrients on physiological, psychological functions or biological activities beyond their established role in growth, development and other normal functions of the body	Certain non-digestible oligosaccharides improve the growth of a specific bacterial group in the gut.
B	*Reduction of disease-risk claims* that are related to a reduction of the risk of a specific disease or condition	Sufficient calcium intake may help to reduce the risk of osteoporosis in later life.

These claims are generated from the scientific evidence based on markers relevant to functional foods. If evidence for the effects of a functional food is based on a marker for target function or biological response, such as changes in body fluid levels of a metabolite, protein or enzyme, then an enhanced function claim might be justified. For a reduced risk of disease claim, however, evidence for the effect of a functional food should be based on an appropriate intermediate endpoint marker of disease, such as the measurement of a biological process that relates directly to the endpoint. Claims of type 4 and A correspond with the American structure-function claim, while the type B claim is similar to the health claim in the USA.

Hence, contrary to Japan and the Unites States, the European Consensus Document prefers a science-based, function-driven approach for functional food science. As the functions and their modulations are universal, in this way a better understanding of the

effects of food components on target functions in the body is obtained (Diplock *et al.*, 1999; Roberfroid, 2000). Recently, a document entitled 'Guidelines for the Scientific Substantiation of Claims on Functional Foods' has been approved by the Council of Europe and is awaiting publication. In the meanwhile, many European Union countries are developing 'Code of Practices', which are generally referring to the European Consensus on Functional Foods (Sanders, 2002).

1.2.5. Perspectives

There is strong evidence that the European market is becoming increasingly ready for functional foods. Consumers now seem to be aware of the relationship between diet and health. They generally agree that the three most important factors contributing to health are diet, exercise and genetic factors (Hilliam, 1998).

However, before being considered an economic challenge, it is and must remain a scientific challenge. Identification and validation of sensitive and reliable markers will be key to adequate assessment of the true effect of foods and their components. These should predict potential benefits or risks relating to a target function in the body. Moreover, the dose necessary to cause a particular effect should be clearly identified and a safety assessment should accompany the demonstration of the effect. It should also be considered that the response to functional foods depends on several factors, including genetics, physiologic state and the composition of the entire diet (Milner, 2000; Roberfroid, 2000).

Food regulations should focus in protecting public health, safety, and interests of the consumers without impeding innovation in the food industry. From the standpoint of food industries, elaborate efforts on research and development are only rewarded if the scientific results are exactly and adequately reflected in the health claims, leading to correct understanding and support of the product by consumers. In an increasingly global economy, these health claims should meet accepted international scientific criteria (Clydesdale, 1997).

1.3. Bioactive peptides

1.3.1. Introduction

Biologically active peptides (BAP) or functional peptides are food derived peptides that in addition to their nutritional value exert a physiological effect in the body. These bioactive peptides are inactive within the original protein, but once released, function as regulatory compounds with hormone-like activity that is based on the inherent amino acid composition and sequence (Meisel, 1997a; Tome, 1998). In this respect, they may present active

ingredients in functional foods and nutraceuticals (Clare and Swaisgood, 2000; Meisel, 1997b).

Bioactive peptides usually contain 2 to 20 amino acid residues per molecule. These peptides can be liberated from the parent protein during gastrointestinal digestion in the body or during food processing (Clare and Swaisgood, 2000). Although these exogenic peptides are less active and specific than their endogenic counterparts, they can be effective after oral administration. They show partial or total resistance to hydrolysis and may either enter peripheral blood intact due to their low molecular size and exert systemic effects, or produce local effects in the gastrointestinal tract (Yoshikawa *et al.*, 2000). Mellander (1950) was the first to name biologically active peptides, when he suggested that casein-derived phosphorylated peptides act as mineral carriers by enhancing vitamin D-independent bone calcification in rachitic infants. The knowledge of bioactive peptides has steadily increased since 1979 and, at present, numerous peptides exhibiting various activities have been reported such as opiate, mineral binding, immunomodulatory, angiotensin I converting enzyme (ACE) inhibitory, antithrombotic and antimicrobial peptides (Clare and Swaisgood, 2000; Meisel, 1998).

Although other animal, as well as plant proteins contain potential bioactive sequences (Dziuba *et al.*, 1999a), milk proteins are currently the main source of a range of bioactive peptides. Milk, a basic food in many diets, has shown to contain many compounds with biological activity and is recognised as a synergistic mixture of multiple interacting factors (German *et al.*, 2002; Xu, 1998). This is somewhat due to the role of milk as an exquisite regulation and communication medium between mother and young (Schanbacher *et al.*, 1997). Most major milk proteins have little or no bioactivity in their native state. Proteolytic digestion of milk proteins, however, releases and activates a plethora of bioactive peptides. Some regions in the primary amino acid sequence of milk proteins contain overlapping bioactive peptide sequences, exerting different biological effects. These regions in the multifunctional bioactive peptides are considered as ‘strategic zones’ that are partially protected from proteolytic breakdown (Figure 1) (Meisel and Bockelmann, 1999).

Recently a database of bioactive peptide sequences has been developed that can be used to identify potential bioactive peptides within a protein sequence. Furthermore, the known cleavage points of proteolytic enzymes like pepsin, trypsin and α-chymotrypsin can be inserted into the database, by which the release of bioactive peptides during digestion of the protein can be predicted (Dziuba *et al.*, 1999b).

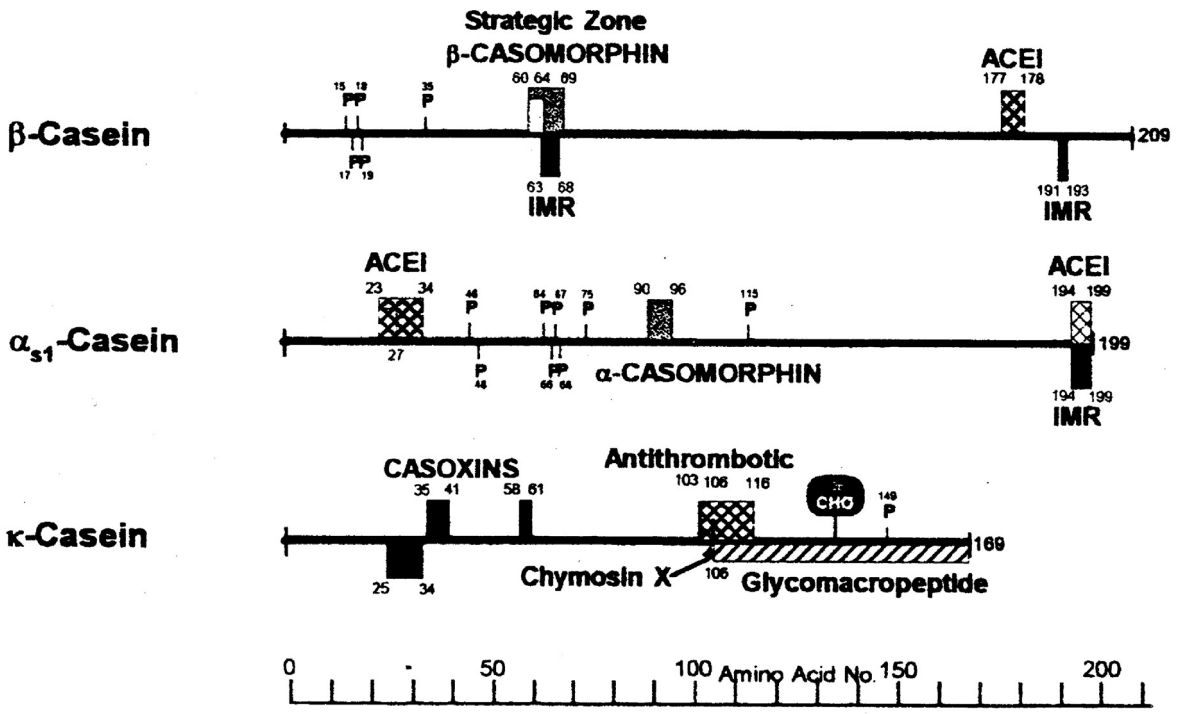


Figure 1. Map of some latent bioactive peptides in bovine β-casein, α_{s1}-casein and κ-casein PP: mineral binding, ACEI: ACE inhibitory, IMR: immunomodulatory region (Schanbacher *et al.*, 1997).

1.3.2. Opiate peptides

Opiate peptides have opiate-like properties and have affinity for an opiate receptor. Their effects are inhibited by naloxone, an opiate antagonist. The individual opiate receptors, which are located in the nervous, endocrine and immune systems, as well as in the myenteric plexus in the gastrointestinal tract, are responsible for specific physiologic effects, e.g. the μ-receptor for emotional behaviour, analgesia and suppression of intestinal motility and respiration, the δ-receptor for emotional behaviour and analgesia, and the κ-receptor for sedation, food intake and analgesia (Froetschel, 1996). The opiate peptides derived from precursor food proteins or exorphins typically have a N-terminal tyrosine and the presence of another aromatic residue like tyrosine or phenylalanine in the third or fourth position (for α-casein and sometimes κ-casein, an arginine residue precedes tyrosine at the N-terminus). The major opiate peptides originate from casein and have been called casomorphins. β-casomorphins, derived from β-casein, have been characterised as μ-type receptor agonists, while α-casomorphins, derived from α-casein, are δ-type receptor agonists. From the bovine whey proteins, α-lactalbumin, β-lactoglobulin and serum albumin respectively the opiate μ-type receptor agonists α-lactorphin, β-lactorphin and serorphin were isolated. The opiate agonistic peptides modulate social behaviour, increase analgesic behaviour, prolong

gastrointestinal transit time, increase the intestinal absorption of amino acids, electrolytes and water, stimulate the secretion of insulin and somastotatin and stimulate food intake (Meisel, 1997b; Xu, 1998). In neonates and newborne calves, β-casomorphin is transported intact through the intestinal wall in the blood, after which it can exert an analgesic effect on the nervous system resulting in calmness and sleep (Clare and Swaisgood, 2000).

Opiate antagonists are found in κ-casein and α_{s1}-casein, the so-called casoxins, with a preference for μ- and κ- type receptors, although their potency is rather low compared to naloxone. Lactoferroxins are antagonists generated from human lactoferrin, with affinity for μ- and κ- type receptors. C-terminally methyl esterified casoxins and lactoferroxins are more active (Clare and Swaisgood, 2000; Meisel, 1997a; Pihlanto-Leppälä, 1999).

1.3.3. Mineral binding peptides

Certain regions of the casein molecules are phosphorylated (α_{s2} > α_{s1} > β > κ). After release, these casein phosphopeptides (CPP) bind mainly Ca^{2+} ions, but also Fe^{2+}, Cu^{2+}, Mn^{2+} and Zn^{2+}. Most CCP contain three serine phosphate clusters, followed by two glutamic acid residues. The specific amino acid composition associated with the phosphorylated binding site influences the degree of calcium binding. CPP are mostly resistant to enzymatic hydrolysis in the gut and most often found in a complex with calcium phosphate. The complex formation of calcium ions results in a higher solubility, which may enhance the intestinal calcium absorption. Hence, CPP may increase the bioavailability of calcium that is required for bone calcification. By the entrapment of certain minerals such as iron, these CPP may also limit the intraluminal availability and therefore the depression of intestinal microbial growth. In addition, CPP may inhibit caries lesions through recalcification of the dental enamel and by inhibiting the adhesion of plaque-forming bacteria. As a result, their application in the treatment of dental diseases and in tooth paste has been proposed (Clare and Swaisgood, 2000; Darragh, 2002; Meisel, 1998).

1.3.4. ACE inhibitory peptides

The multifunctional enzyme ACE plays an important role in the renin-angiotensin system, which regulates both arterial blood pressure as well as salt and water balance. In the cardiovascular system, ACE converts angiotensin I to angiotensin II, a potent vasoconstrictor, and degrades bradykinin, a vasodilator. Therefore, inhibition of ACE has been shown to result in an antihypertensive effect in hypertensive humans and animals. ACE inhibitory peptides derived from casein, casokinins, and whey, lactokinins, have been isolated (Fitzgerald and Meisel, 2000). These peptides are further discussed in section 2.3.2.

1.3.5. Immunomodulatory peptides

Immunomodulatory milk peptides affect both the immune system and cell proliferation responses. Work with animal cell lines has shown that peptides derived from whey and casein stimulate phagocytosis of human and murine macrophages and protect against *Klebsiella pneumonia* infection in mice (Migliore-Samour *et al.*, 1989). Lactoferricin B, obtained by pepsin hydrolysis of lactoferrin, promotes phagocytic activity of human neutrophils (Mijauchi *et al.*, 1998). Both stimulatory and suppressive immune responses, or increased and decreased proliferation, of human lymphocytes to milk derived peptides have been observed. Feeding peptide fractions from a *Lactobacillus helveticus* milk ferment to mice increases the number of IgA producing cells in the intestine and decreases the size of subcutaneously implanted fibrosarcomas (LeBlanc *et al.*, 2002). Immunomodulating milk peptides have also shown to inhibit the development of infections in patients with pre-AIDS (Meisel, 1998).

In general, the mechanisms by which these immunomodulatory peptides exert their immunopotentiating effects are currently unknown, although opiate receptor pathways have been suggested (Clare and Swaisgood, 2000; Meisel, 1998).

1.3.6. Antithrombotic peptides

Mechanisms involved in the clotting of milk (interaction of κ-casein with chymosin) bear a remarkable resemblance to the processes involved in blood clotting (interaction of fibrinogen with thrombin) and sequence homologies exist in the fibrinogen γ-chain and κ-casein. As a result, peptides derived from casein glycomacropeptide (CMP), the soluble C-terminal fragment of κ-casein after splicing by chymosin, have antithrombotic activity. These casoplatelines compete with the γ-chain of fibrinogen for the platelet receptors, thus inhibiting platelet aggregation (Clare and Swaisgood, 2000; Darragh, 2002).

1.3.7. Antimicrobial peptides

Antimicrobial peptides have been derived from the whey protein lactoferrin, an iron-binding glycoprotein. Lactoferricin, obtained from a peptic digest of lactoferrin, has even more potent bactericidal properties than the undigested parent protein, suggesting that its much smaller size may facilitate access to target sites on the microbial surface. The antimicrobial peptides isracidin and casocidin, are derived from α_{s1}- and α_{s2}-casein respectively. The antimicrobial activity of all these peptides seems to be correlated with their net positive charge that binds to the negatively charged phospholipids of the cell membrane and their large content of hydrophobic residues, both increasing cell membrane permeability

and finally killing off sensitive microorganisms. Antimicrobial peptides with bactericidal activity against various Gram-positive (*Bacillus*, *Listeria* and *Streptococcus*) and Gram-negative (*Escherichia coli*, *Klebsiella*, *Salmonella*, *Proteus* and *Pseudomonas*) microorganisms have been identified. In addition, antifungal properties (*Candida albicans*) have been demonstrated (Clare and Swaisgood, 2000; Darragh, 2002).

1.3.8. Miscellaneous peptides

Antioxidative peptides and their synthetic analogues inhibit *in vitro* the oxidation of unsaturated fatty acids. They scavenge free radicals and form complexes with the metal ions catalysing free radical reactions. Tyrosine and histidine, which are susceptible to oxidation, are typical components of these peptides from soybean. Methionine, lysine and tryptophan have similar properties. The antioxidative capacity of peptides is enlarged by the presence of proline residues in their sequence, which enhances the interaction with unsaturated fatty acids (Dziuba *et al.*, 1999a).

Food derived peptide sequences may be involved in the modulation of cancer cell function and this sometimes overlaps with an immunomodulation function (LeBlanc *et al.*, 2002). Bioactive peptides in yoghurt preparations both suppress proliferation and increase apoptosis of cancer cells in animal and human cell lines (Ganjam *et al.*, 1997), whereas lactoferricin can inhibit metastasis and angiogenesis. Peptides resulting from the fermentation of whey by *Lactobacillus helveticus* L89 may contribute to the antimutagenic effect observed in the Ames test (Matar *et al.*, 1997). Fragments of the hen egg ovomucin cause remission of fibrosarcoma cancer in mice after intratumour injection (Watanabe *et al.*, 1998). Cell growth-promoting peptides are also known: a fragment of lactoferrin and casokinins stimulate DNA-synthesis in animal epithelial cells (Darragh, 2002).

Several peptides contract smooth muscles, which can be investigated *in vitro* using fragments of pig guinea pig ileum. Examples are albutensin A from bovine serum albumin, β-lactotensin from β-lactoglobulin and oryzatensin from rye. It is suggested that the action on smooth muscles and on immune cells is mediated by the same receptor (Dziuba *et al.*, 1999a; Shah, 2000).

Furthermore, several peptides are known as inhibitory compounds to various enzymes like prolyl endopeptidases, which may have an antiamnesic effect (Dziuba *et al.*, 1999a), and amino- and endopeptidases of lactic acid bacteria and *Pseudomonas fluorescens* (Smacchi and Gobbetti, 1998).

Some peptides exert an antihypertensive effect by other mechanisms than ACE inhibition, but these are discussed in section 2.5.

1.4. Whey and pea protein

1.4.1. Whey protein

Milk proteins consist of 80% casein and 20% whey. Whey proteins remain in milk serum or whey after precipitation of casein by acid at pH 4.6 or by rennet at pH 6.7. Compared with casein, whey protein is more heat-sensitive, less calcium-sensitive and can engage in thiol-disulfide interchanges to form oligomeric structures. The major whey proteins are β-lactoglobulin, α-lactalbumin, bovine serum albumin, immunoglobulins and proteose peptones (Table 3). β-lactoglobulin and α-lactalbumin are synthesised in the mammary gland of the cow, while bovine serum albumin and immunoglobulins are derived from the blood. Casein-derived polypeptides include the proteose peptone fraction, present in acid and rennet or sweet whey, and the glycomacropeptides, present only in rennet whey (Belem *et al.*, 1999; Eigel *et al.*, 1983; Kilara and Harwalkar, 1996; Kinsella and Whitehead, 1989; Wong *et al.*, 1996).

The main whey protein in milk, β-lactoglobulin, has a very hydrophobic area that is quite effective in binding retinol and other hydrophobic molecules. Therefore, it is speculated that its biological function is related to vitamin A transport. The B variant is predominant in Western cattle. Between pH 3.5 and 5.2, at low temperatures and high protein contents, β-lactoglobulin associates to form an octamer of 147 kDa. Between pH 5.2 and 7.5, including the pH of milk, β-lactoglobulin tends to be found as a dimer of 36.7 kDa. At very low and high pH, β-lactoglobulin exists as a monomer. β-lactoglobulin contains one free thiol group per molecule, which is of great importance in reactions with other proteins, notably κ-casein and α-lactalbumin.

In the synthesis of lactose, α-lactalbumin interacts with galactosyltransferase by increasing its affinity for the transfer of galactose to glucose. α-lactalbumin is a calcium metalloprotein and it is stabilised against heat denaturation and aggregation by calcium. Three genetic variants are identified, although only variant B is found in the milk of western breeds. The amino acid sequence of α-lactalbumin is very similar to that of lysozyme. Recently, α-lactalbumin was shown to regulate the growth of cancerous cells *in vitro* (Sternhagen and Allen, 2001).

Bovine serum albumin (BSA) from bovine milk is identical to the blood serum molecule and is presumed to be a leakage molecule. In the blood and in milk as well, BSA is a carrier for insoluble fatty acids, which protect the molecule against heat. It has specific binding sites for hydrophobic molecules and one free thiol group.

Table 3. Whey proteins and their properties in milk (Eigel *et al.*, 1983; Kilara and Harwalkar, 1996; Kinsella and Whitehead, 1989; Wong *et al.*, 1996).

Component	Approximate % of total whey protein	Iso-electric point pI	Number of amino acids	Molecular weight (kDa)
β-lactoglobulin	50	5.2	162	36.7 (18.3)
α-lactalbumin	18	4.8	123	14.2
bovine serum albumin (BSA)	5	4.7	582	66.3
immunoglobulin G_1	8	5.5 - 6.8		160
immunoglobulin G_2	0.8	7.5 - 8.3		150
immunoglobulin M	0.8			900
immunoglobulin A	1.4			400
proteose peptone	15	3.3 - 3.7		4.1 - 20
lysozyme	1	9.5	130	18
lactoferrin	1	8.7	691	76.5

The immunoglobulins in milk provide passive immunity for the neonate. There are four classes found in milk: IgG_1, IgG_2, IgA and IgM. All of these molecules have a similar basic structure composed of two light chains (Lc) with molecular weights of 20-25 kDa and two heavy chains (Hc), having molecular weights of 50-70 kDa, linked by disulfide bridges. They are very thermolabile. IgG_1 is the major immunoglobulin in milk. IgA is the second most abundant immunoglobulin in milk, where it exists as a dimer and is usually bound to the secretory component, an epithelial glycoprotein of 83 kDa. IgM exists as a pentamer in milk.

The proteose peptone fraction is defined as a mixture of phosphoglycoproteins that are heat stable at 95°C for 20 minutes at pH 4.7 and are precipitated by 12% (w/v) trichloroacetic acid. This fraction is usually designated as component 3 (dimer 40.8 kDa), 5 (13 kDa), 8-fast (4.1 kDa) and 8-slow (9.9 kDa) in ascending order of electrophoretic mobility at pH 8.6. Proteose peptone component 3 is found in whey only and not in casein.

Some minor whey proteins may have important biological functions. Lysozyme is considered a significant component of the antibacterial system of milk, possibly affecting the general immune system as well. Lactoferrin, an ironbinding glycoprotein, has an antimicrobial, antiviral and immunomodulatory function (Shah, 2000; Steijns, 2001).

1.4.2. Pea protein

Pea protein is mainly composed of water soluble proteins: globulins, which are also soluble in salt solutions, and albumins. The insoluble protein is poorly characterised up to now. Pea protein is a storage seed protein that serves as nitrogen source for the developing embryo during germination (Guéguen, 2000). Pea proteins are distinguished from soy proteins by the presence of sulphur-rich albumins.

Table 4. Pea proteins and their properties in pea (Guéguen, 2000; Page and Duc, 1999).

Component	Approximate % of total pea protein	Iso-electric point pI	Molecular weight (kDa)
legumin (11S)	28	5.6 - 6.1	380
vicilin & convicilin (7S)	32	5.3 & 5.4 – 6.1	170 & 280
albumins (2S)	25		
insoluble protein	15		

Globulins are products of a multi-gene family with different patterns of synthesis and accumulation of these proteins during seed development and the formation of heteropolymers. Therefore, the different genes of a globulin usually have high homologous sequences (Gatehouse *et al.*, 1984). As in other leguminous seeds, pea globulins are composed of two main families, legumin and vicilin, which belong respectively to the 11S and 7S seed storage protein classes. Legumin, homologous to soybean glycinin, is a closely packed hexameric protein. The monomer is constructed from two subunits, the acidic α (40 kDa) and basic β (20 kDa) polypeptides, linked by disulfide bonds. The more hydrophilic α subunits are situated at the outside, while the β subunits constitute the hydrophobic heart of the polymeric protein. Legumins are very variable in terms of composition of subunits $\alpha\beta$: in total, 22 different α polypeptides and 11 β polypeptides have been identified. About 10 genes coding for $\alpha\beta$ precursors are known. Vicilin is characterised by a trimeric structure, like soybean conglycinin and bean phaseolin. It does not contain cysteine and therefore the trimeric structure is only stabilised by weak interactions. Pea vicilins are very complex structures in respect of their subunit composition. Up to eight classes of subunits with molecular weights from 12 to 50 kDa are known. They seem to be produced by proteolysis from a 50 kDa precursor, which can be encoded by 11 different genes. Vicilins and legumins have evolved from the duplication of the same ancestral gene, but in legumins a novel N-terminal domain has been recruited somewhere in evolution (Gibbs *et al.*, 1989). In addition, the trimeric protein convicilin is also present in small quantities. Convicilin subunits

have a molecular weight of about 71 kDa and differ only from vicilin by the insertion of a 121 amino acid sequence near the N-terminus of the protein (Bown *et al.*, 1988; Croy *et al.*, 1980). This inserted sequence is very hydrophilic and is of similar amino acid composition to the sequences found near the C-terminal region of the α subunit in legumin. Up until now, the 7S proteins remain poorly characterised (Gatehouse *et al.*, 1984; Guéguen, 2000; Page and Duc, 1999; Thompson *et al.*, 1991).

Albumins mainly comprise proteins with a biological function in the seed, like enzymes, enzyme inhibitors and plant defense proteins. Consequently, this protein fraction, which is characterised by a high lysine and sulphur amino acid content, is very heterogeneous. It contains PA1 albumin, a sulphur-rich protein dimer of 10 kDa, including the two polypeptides PA1a of 6 kDa and PA1b of 4 kDa. This fraction is suggested to be a source of sulphur containing amino acids for the plant embryo. The larger dimer PA2 albumin of 53 kDa is constituted of two similar polypeptides PA2a and PA2b of 26 kDa (Croy *et al.*, 1984). The physiological role of this albumin fraction is not yet recognised.

2. THE ANGIOTENSIN I CONVERTING ENZYME

2.1. The renin-angiotensin system and the kallikrein-kinin system

The renin-angiotensin system is a complex regulatory system that plays a key role in maintaining blood pressure homeostasis, as well as fluid and salt balance in mammals. In the classical pathway of the renin-angiotensin system, activation of a cascade of enzymatic reactions results in the generation of the main effector molecule angiotensin II, which acts both as a systemic and a locally generated paracrine or autocrine effector peptide (Figure 2). The protease renin, which is secreted predominantly in the kidney, cleaves the liver-derived precursor peptide angiotensinogen into the decameric peptide angiotensin I. Angiotensin I is further hydrolysed into the vasoconstrictor octapeptide angiotensin II by the angiotensin I converting enzyme (ACE, EC 3.4.15.1). ACE also inactivates the vasodilator peptides bradykinin and kallidin, which explains why ACE is also termed kininase II (Figure 3). Hence, activation of the renin-angiotensin system mainly results in a systemic vasopressor response by blocking the hypotensive kinin-bradykinin pathway and by generation of the vasoconstrictor angiotensin II (Ganong, 1997b).

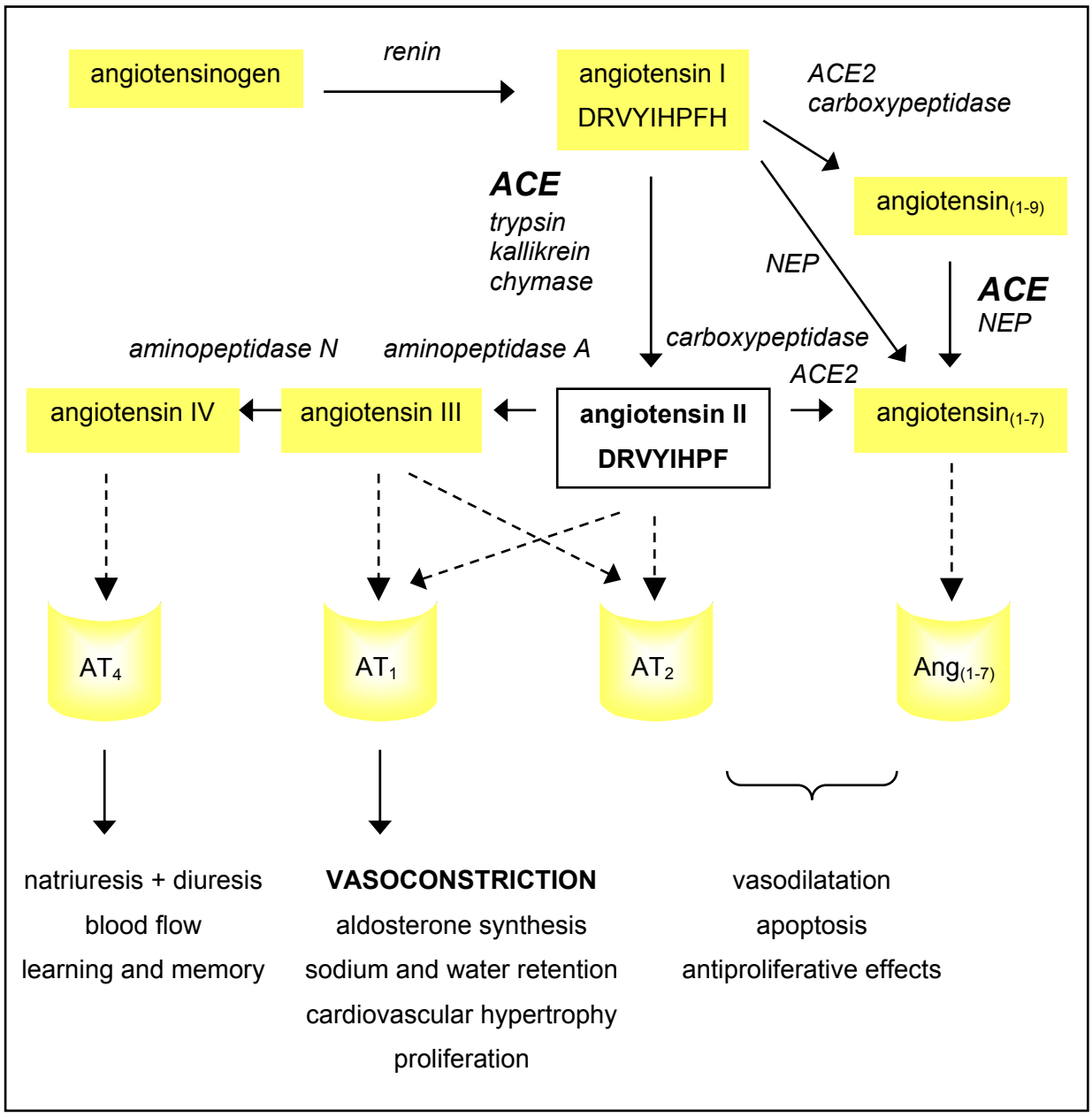


Figure 2. The renin-angiotensin system: the interaction of the different biological active peptides with their receptors and effects (ACE = angiotensin I converting enzyme, ACE2 = human homologue of ACE, NEP = neutral endopeptidase).

However, the renin-angiotensin system is far more complex than initially thought.

In addition to the circulating hormone system, the renin-angiotensin system also serves as a local tissue hormone system (Campbell, 1987; Johnston, 1992). All components of the renin-angiotensin system have been identified in a variety of tissues like the brain, the epithelial cells of the intestine, kidneys and eye, the reproductive tract, the cardiovascular system and fibroblasts and macrophages. In the brain for example, ACE assists in neuropeptide processing. In the intestine, ACE aids in the digestion of oligopeptides and angiotensin II controls sodium and water absorption (Levens, 1985; Yoshioka *et al.*, 1987).

ACE is also expressed on fibroblasts and in differentiated macrophages and T-lymphocytes and may therefore be important in the inflammatory process and the repair of tissues. In the reproductive tract, ACE has a crucial role in fertility.

Angiotensin II mediates a broad array of physiological and pathophysiological effects by binding to specific cell membrane receptors. It exerts a direct vasoconstrictor action on the vascular smooth muscle cells and stimulates the synthesis and release of aldosterone in the adrenal glands, which results in sodium and water retention. Furthermore, it suppresses the release of renin in the kidney. In the peripheral nervous system it stimulates the release of adrenaline and noradrenaline, thereby enhancing sympathic activity of (heart and arterial) muscles. In the central nervous system, angiotensin II stimulates the release of vasopressin and antidiuretic hormone and creates a feeling of thirst. Since angiotensin II acts as a growth regulator in different cell types, it stimulates (cardiac and vascular) hypertrophy and can mediate morphological changes in organs. Moreover, angiotensin II stimulates NO and prostaglandin release from endothelial cells, thus antagonising the vasoconstrictor actions of the peptide. In some tissues, angiotensin II is synthesised by non-ACE enzymes, for example by chymases in the heart (Johnston, 1992; Millatt *et al.*, 1999).

So far, two distinct types of angiotensin II receptors have been characterised, which are heterogeneously distributed in the human body. The AT_1 receptor is predominantly expressed in the kidneys, adrenal glands, cardiovascular system and brain and it is to this receptor that the regulatory actions of angiotensin II on blood pressure and salt-water balance have been attributed. The AT_2 receptor is present at high density during fetal development, while in the adult it is only significantly found in the adrenal glands, uterus, ovary, endothelial cells and brain. The AT_2 receptor is thought to counterbalance effects mediated by the AT_1 receptor (Millatt *et al.*, 1999). It is suggested that AT_1 and AT_2 receptors interact physically with each other to antagonise their respective actions (AbdAlla *et al.*, 2001).

The renin-angiotensin system involves more angiotensin-derived mediators than previously recognised: both angiotensin I and II are further metabolised to other biologically active peptides with distinct properties, which can either enhance or compensate the effects of angiotensin II: angiotensin III, angiotensin IV and angiotensin$_{(1\text{-}7)}$ (Eriksson *et al.*, 2002; Turner and Hooper, 2002).

Recently, two alternative, independent genomics-based strategies have led to the discovery of a homologue of human ACE, termed ACE2 or ACEH, which functions as a carboxymonopeptidase with a preference for C-terminal hydrophobic or basic residues and appears to negatively regulate the activated renin-angiotensin system (Donoghue *et al.*, 2000; Tipnis *et al.*, 2000).

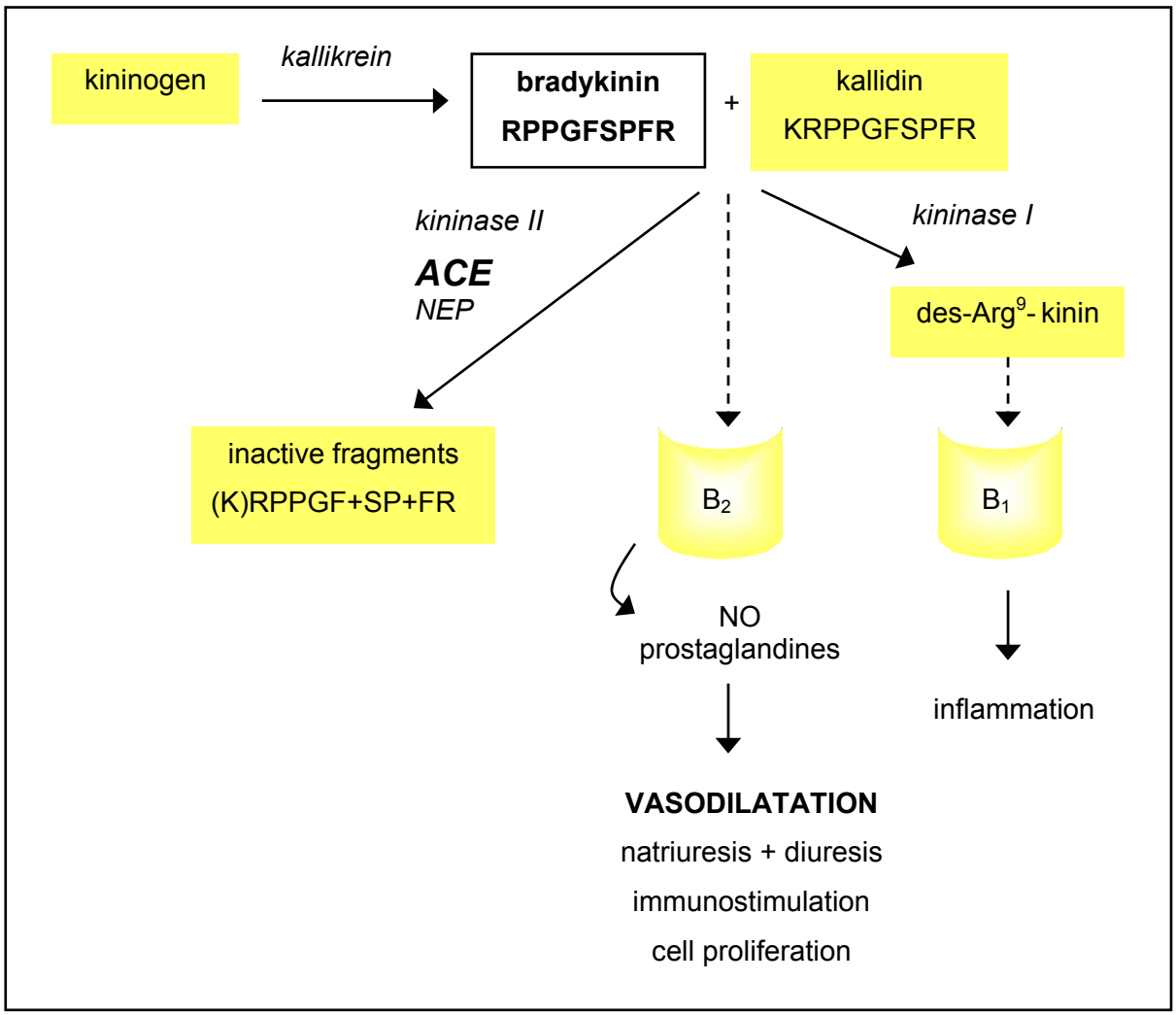


Figure 3. The kallikrein-kinin system: the interaction of the different biological active peptides with their receptors and effects (ACE = angiotensin I converting enzyme, NEP = neutral endopeptidase).

ACE plays also a functional role in the kallikrein-kinin system.

The kinins bradykinin and kallidin (Lys-bradykinin) are cleaved from kininogens by kallikreins. Tissue kallikrein releases kallidin from low-molecular-weight kininogen and plasma kallikrein releases bradykinin from high-molecular-weight kininogen. Kinins are degraded by kininases: kininase I type enzymes cleave the C-terminal arginine residue (carboxypeptidase N and M) and kininase II type enzymes remove the C-terminal dipeptide Phe-Arg (ACE and neutral endopeptidase). The actions of kinins are mediated via B_1 and B_2 receptors. Apparently, the B_1 receptors are not constitutively present, but are induced by certain environmental factors, like in inflamed and injured tissues. Des-Arg^9-kallidin is an important agonist, in contrast to bradykinin, which is essentially inactive at the B_1 receptor. Most of the actions of bradykinin and kallidin are mediated through B_2 receptors, which are expressed in most tissues. Kinins are potent vasoactive peptides that cause vasodilatation

and increased vascular permeability. They are responsible for the cardinal signs of inflammation, produce pain and have the ability to release cytokines from monocytes. In addition, they lower systemic blood pressure, increase capillary permeability and produce natriuresis. Kinins also stimulate the secretion of renin from the kidney and the release of vasopressin from the brain. In the nervous system, bradykinin is involved in the central regulation of the blood pressure, nociception and diuresis, and it increases neuronal excitability (Cassim *et al.*, 2002). The kallikrein-kinin system and the renin-angiotensin system are not only linked by ACE, but also by the physical interaction between the AT_1 and the B_2 receptor (Abdalla *et al.*, 2000).

In addition to the renin-angiotensin and the kallikrein-kinin systems, Moskowitz (2002) launched a revolutionary theory that ACE functions at the start of a signalling pathway common to major diseases that are otherwise unrelated, including cardiovascular disease, cancer and psychiatric disease. Although ACE is undoubtedly the primary regulator of angiotensin II production and bradykinin inactivation, it is clear that other pathways and enzymes also modulate these processes. Furthermore, ACE might have additional physiological functions, which are even distinct from its enzymatic activity. It appears that the complexity of the renin-angiotensin system guarantees well-tuned adaptation of the blood supply of the body compartments and organs to all physiological conditions (Eriksson *et al.*, 2002; Turner and Hooper, 2002).

2.2. Properties of ACE

2.2.1. Physical and chemical structure of ACE

The zinc metallopeptidase ACE is anchored to the plasma membrane through a single C-terminal transmembrane domain and is oriented in such a way that the catalytic sites are exposed on the extracellular surface of the cell (Figure 4). In mammals, two distinct ACE isoenzymes have been described, an abundant *somatic form* (170 kDa) found on the endothelial surfaces of the lungs and on brush border membranes of kidneys, intestine, placenta and choroid plexus, and the smaller *germinal or testicular form* of ACE (100 kDa) found only in the testis (Soubrier *et al.*, 1993a). Both ACE isoforms function as ectoenzymes which hydrolyse circulating peptides. While germinal ACE has a crucial role in fertility, tissue-bound somatic ACE controls both blood pressure and renal structure and function. A *soluble form* of ACE, which is derived from the membrane form through the action of a secretase, is also present in serum and other body fluids, but its biological significance is not clarified (Parvathy *et al.*, 1997; Turner and Hooper, 2002). All forms of ACE are heavily glycosylated and it is suggested that various patterns of glycosylation modulate the substrate specificity of

ACE (Orth *et al.*, 1998). Consequently, ACE is one of the glycoproteins that comprise the endothelial cell's unstirred layer.

Somatic ACE is composed of two highly homologous domains, the N-domain and the C-domain, suggesting a gene duplication event in the course of evolution. Each domain contains the typical zinc-binding motif, His-Glu-X-X-His, at the active site, which is found in many zinc peptidases. The two histidine residues provide two of the three zinc-coordinating ligands and the carboxyl group of glutamate is the base donor in the catalytic reaction. The third zinc co-ordinating residue is another glutamate. The testicular ACE sequence corresponds to the C-domain of somatic ACE and therefore contains only one active site and one zinc-binding motif (Corvol *et al.*, 1995; Soubrier *et al.*, 1993a).

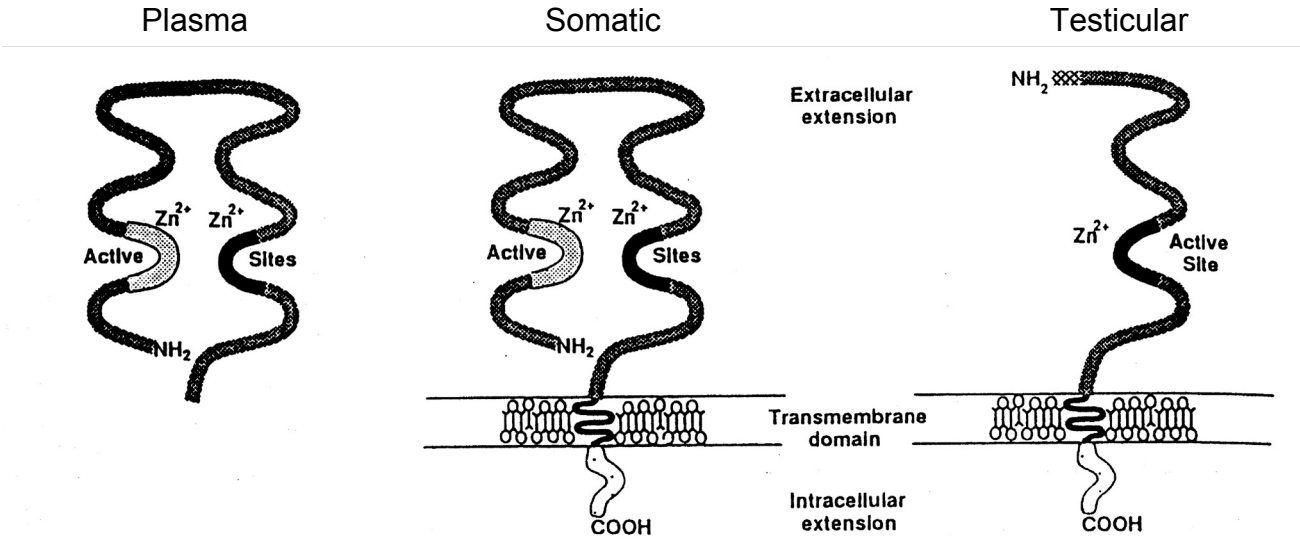


Figure 4. Structure and conformation of the angiotensin I converting enzyme derived from plasma, somatic cell origin and testis, showing the active catalytic sites, zinc dependency and the N- and C-terminal ends (Johnston, 1992).

2.2.2. Molecular structure of ACE and phylogenesis

The somatic and germinal ACE mRNA are transcribed from the same gene, *ace*, located on chromosome 17 in humans, using alternate promoters. The somatic promoter is active in several cell types, whereas the germinal promoter is only active in a stage-specific manner in male germinal cells. The human ACE gene contains 26 exons. The somatic ACE mRNA is transcribed from exon 1 to 26, but exon 13 is spliced during maturation of the somatic ACE transcript. The germinal mRNA is transcribed from exon 13 to exon 26. Exons 4-11 and 17-24, encoding the two homologous domains of the ACE molecule, are highly similar, both in

size and sequence. This further supports the duplication of an ancestral ACE gene (Soubrier *et al.*, 1993a).

The duplication of the ACE gene occurred early in evolution, since from the origin of the Chordata on, the duplicated ACE form is found. Orthologs of ACE are expressed in every kingdom, including Archaea and Eubacteria. High levels of ACE are found in the male reproductive tracts of several insect species, which indicates that a role for ACE in reproduction is evolutionary widespread. ACE is also present in other parts of the insect organism and has shown to be important in embryogenesis. It is also suggested that ACE could serve as prohormone convertase in insects. Together with the fact that human somatic ACE is involved in the processing of neuropeptides further implies an evolutionarily conserved role for ACE in the biosynthesis of some regulatory peptides. Unlike mammalian somatic ACE, the ACE like enzymes of the housefly *Musca domestica* and the fruitfly *Drosophila melanogaster* are single-domain proteins without a membrane anchor. However, there is still no evidence for the existence of angiotensin I and bradykinin-like peptides in insects. Although a single copy of an ACE-like gene is present in the *Caenorhabditis elegans* genome, no enzyme ACE activity has been observed (Isaac *et al.*, 2000). Therefore, it is suggested that ACE might have an additional, non-catalytic function. Overall, information on invertebrate ACE will increase our knowledge about the functions of the evolutionarily conserved human ACE (Turner and Hooper, 2002).

2.2.3. Enzymatic properties of ACE

The primary specificity of ACE is in acting as dipeptidyl carboxypeptidase that removes the C-terminal dipeptide from substrates that have a free carboxyl terminus. It preferentially cleaves substrates with hydrophobic amino acids in the antepenultimate position and has little affinity for substrates with C-terminal dicarboxylic amino acids (Glu, Asp) or with proline in the penultimate position (Cheung *et al.*, 1980). By this mechanism, it catalyses the cleavage of angiotensin I to the potent vasoconstrictor peptide angiotensin II and inactivates the vasodilator peptides bradykinin and kallidin (Corvol *et al.*, 1995). ACE also hydrolyses the inactive angiotensin$_{(1\text{-}9)}$ peptide into the vasodilator metabolite angiotensin$_{(1\text{-}7)}$ (Eriksson *et al.*, 2002). Although ACE hydrolyses a wide range of substrates, bradykinin is the most favourable substrate for ACE and displays a Michaelis Menten constant K_m that is at least ten times lower than for angiotensin I (Bunning *et al.*, 1983; Corvol *et al.*, 1995).

In addition, it degrades enkephalins, C-terminal extended pro-enkephalins and neurotensin. ACE may act as endopeptidase on substrates that are amidated at the carboxyl terminus like substance P and the luteinising hormone releasing hormone (LHRH). It also releases the C-terminal tripeptide from des-Arg^9-bradykinin. Moreover, ACE cleaves the

tripeptide from the blocked N-terminus of LHRH. However, the tripeptide amide is not invariably released from C-terminal blocked substrates; cholecystokinin-8 and various gastrin analogues are hydrolysed by ACE to release the dipeptides as the initial and major products. While these enzymatic activities have been observed *in vitro*, there is no convincing evidence that they bear any physiological relevance, except maybe for substance P in the brain (Erdös and Skidgel, 1987; Soubrier *et al.*, 1993a).

Chemical modification experiments have identified glutamic acid, tyrosine and arginine as catalytic components of the active site (Figure 5). The substrate interacts through its carbonyl group with the essential zinc atom to facilitate the nucleophilic attack of the carboxyl group of glutamic acid. Subsequent addition of a water molecule completes the cleavage of the peptide bond. Tyrosine donates a proton to the scissile peptide NH group, while specificity is determined by interaction of the terminal carboxyl group with arginine (Bünning, 1987). Crystallisation of the ACE enzyme needs to confirm this model.

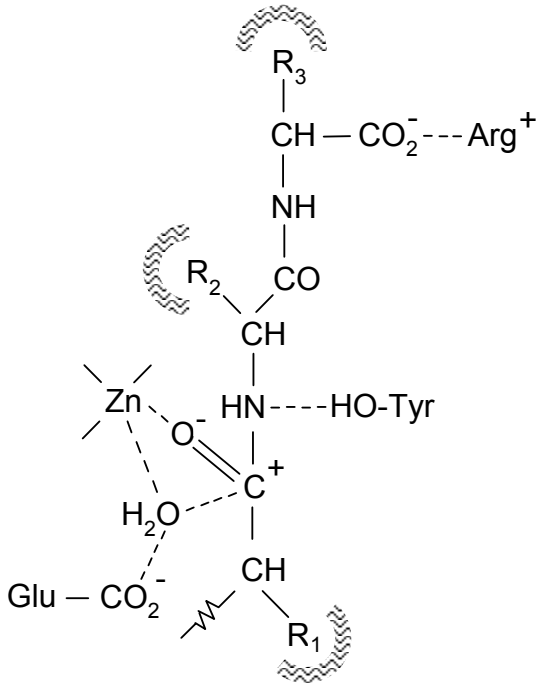


Figure 5. Hypothetical model of the active site of ACE and the interaction with a peptide substrate (Bünning, 1987).

Two moles of zinc are required per mole ACE due to the two active sites. Chloride ions exert an activating effect on the catalytic activity of ACE. Bradykinin is hydrolysed in the absence of chloride ions, although the hydrolysis of angiotensin I requires the presence of chloride ions. The kinetics of chloride activation are rather complex, being both substrate- and pH-dependent. By binding to a lysine residue in the region of the active site, chloride

changes the conformation of ACE, thereby improving substrate binding (Bunning, 1983; Corvol *et al.*, 1995).

Both the N- and C-domain of ACE are catalytically active and appear to function independently. However, the two domains have some differences in substrate specificity and different patterns of chloride activation. For example, the N-domain cleaves the N-terminal tripeptide of LHRH much faster than does the C-domain. The haemoregulatory peptide N-acetyl-Ser-Asp-Lys-Pro is the most specific substrate identified to date for the N-domain, but a specific substrate for the C-domain has not yet been found. The two domains hydrolyse angiotensin I and bradykinin at a comparable rate. However, the C-domain requires high concentrations of Cl^- for optimal activity, a property that seems to be conferred by a single arginine residue in this domain. Moreover, the two domains differ in their affinities to bind several ACE inhibitors: enalapril for example, preferentially binds to the C-domain while ramipril can block both domains. The N-domain of ACE contains a deeply recessed active site and therefore the corresponding zinc atom is less accessible to inhibitors (Corvol *et al.*, 1995; Turner and Hooper, 2002; Wei *et al.*, 1991).

2.3. ACE inhibition

2.3.1. ACE inhibitory drugs

ACE inhibitors were first discovered in snake venom of *Bothrops jararaca* in 1970. These inhibitory drugs are oligopeptides with 5 to 13 amino acids per molecule. The nonapeptide teprotide, Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro, named for the four proline residues that help to confer biological stability, is the most active of the snake peptides *in vivo*. However, a second snake venom peptide, bradykinin-potentiating peptide 5a (BPP_{5a}), Glu-Lys-Trp-Ala-Pro, or more precisely its Phe^3 analogue, is the legitimate progenitor of the huge family of ACE inhibitors that has proliferated.

Structure-activity studies with analogues of snake venom peptides indicate that the C-terminal tripeptide residue makes the greatest contribution to their overall binding to the active site of ACE. The optimal C-terminal sequence for active site binding is Trp-Ala-Pro, as encountered in BPP_{5a}, even though this sequence allows the pentapeptide to be cleaved rapidly *in vivo*, under some conditions by ACE itself, thus preventing its usefulness as an antihypertensive drug. Therefore, the tryptophan side chain has usually been replaced by that of the more stable amino acid phenylalanine in competitive inhibitors of ACE. As a result, all clinical important ACE inhibitors are structurally related to the tripeptide Phe-Ala-Pro. These amino acids are shown to produce an optimal interaction with the subsites S_1, S'_1 and S'_2 at the active site of the ACE enzyme (Figure 6) (Cushman and Ondetti, 1999).

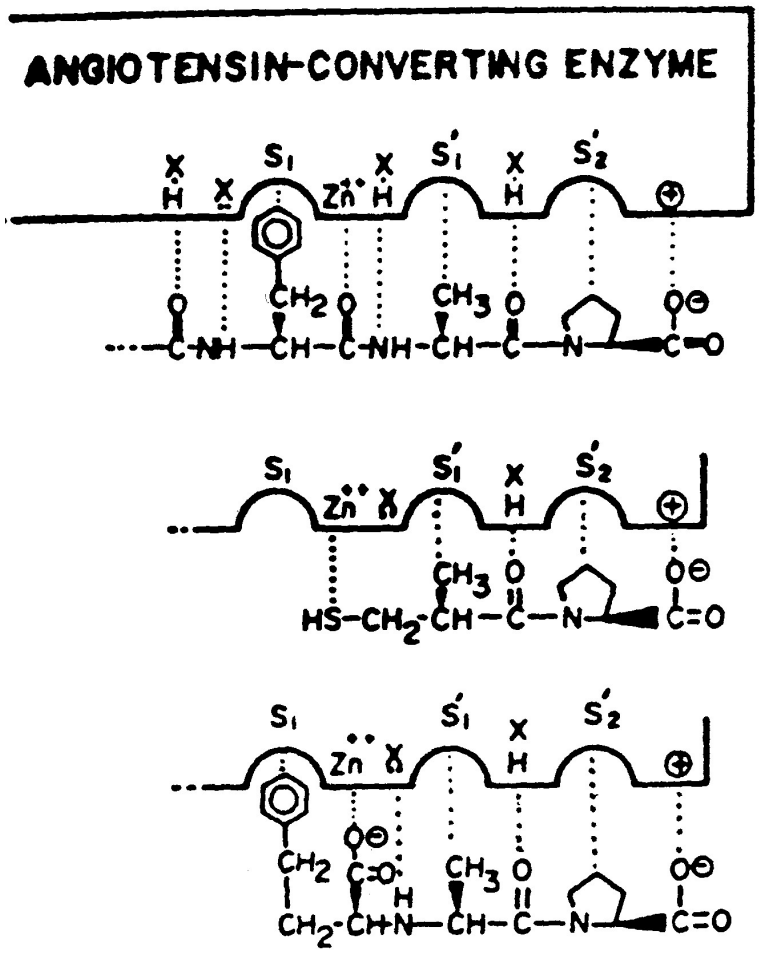


Figure 6. Hypothetical binding of competitive inhibitors to the active site of ACE. The subsites S_1, S'_1, S'_2 interact with side chains of terminal, penultimate and antepenultimate amino acids or residues of inhibitors. The residue X-H donates a hydrogen bond and X accepts a hydrogen bond (Cushman *et al.*, 1987).

The search for drugs that resisted gastrointestinal digestion after oral administration and that showed a greater affinity for the active site of ACE resulted in the design of the dipeptide analogue inhibitors. Captopril is the major representative of this group. It can be considered an analogue of the C-terminal dipeptide Ala-Pro of the snake venom peptide BBP_{5a}, in which the amino function of alanine is replaced by a sulfhydryl function that is able to strongly interact with the catalytically essential zinc ion of the enzyme. In addition, the terminal carboxyl group interacts with the positive charge of arginine at the active site, the carbonyl group with the hydrogen bond donor tyrosine, the heterocyclic ring of the prolyl residue with a hydrophobic pocket of the enzyme S'_2 and the methyl group with the S'_1 subsite (Figure 6) (Bünning, 1987; Cushman *et al.*, 1987).

Most of the newer ACE inhibitors are analogous to the C-terminal tripeptide of the BBP_{5a} Phe^3 analog, and therefore named tripeptide analogue inhibitors. Enalaprilat is the active free acid form of the antihypertensive drug enalapril, which is administered as an ester prodrug to enhance intestinal absorption (Johnston *et al.*, 1986). Enalaprilat binds to the active site of ACE by employing most of the interactions of the C-terminal residue of BPP_{5a}, while replacing the weak interaction of the amide carbonyl by a strong interaction of its carboxyl function with the zinc ion of the enzyme (Figure 6). The carboxyl function also prevents the drug from cleavage by ACE. Under physiologic conditions *in vitro*, enalaprilat is more active than captopril. Another class of tripeptide analog inhibitors employs the hydroxyphosphinyl function as the zinc-binding ligand and is represented by phosphinic acids, phosphonamidates and phosphonic acids.

Most of the ACE inhibitors discussed before are remarkably active *in vitro*. Although newer modifications have led to compounds with increasing inhibitory potency, often only increased oral effectiveness, increased duration of action, an alternate route of excretion or other altered pharmacokinetic properties were obtained. Lisinopril, an analogue of the tripeptide sequence Phe-Lys-Pro, is similar to enalaprilat, but has useful oral activity without esterification. In ramiprilat, which is more potent than enalaprilat, the C-terminal proline is replaced by a proline surrogate, azabicyclo-octane carboxylic acid. Ramiprilat is also dosed as an ethyl-ester derivatized prodrug ramipril (Cohen, 1985; Cushman *et al.*, 1987; Cushman and Ondetti, 1999). The potencies of some of these ACE inhibitory drugs are displayed in Table 5.

ACE inhibitory drugs are considered as competitive and reversible, slow and tight-binding inhibitors of ACE (Bünning, 1987). These drugs are now used clinically to treat hypertension, congestive heart failure and myocardial infarction, endothelial dysfunction and renal disease, including diabetic nephropathy (Taylor, 2001; Turner and Hooper, 2002). Moreover, ACE inhibitors potentiate the effect of bradykinin and its analogues on their B_2 receptors independently of blocking peptide metabolism (Erdös *et al.*, 1999). Clinical trials are under way to test new uses for ACE inhibitors like anti-inflammatory, anti-oxidative and anti-tumour effects and improvement of cognitive deficiencies (Bunk, 2002). Adverse side affects related to ACE inhibitors include irritating cough, excessive drops in blood pressure, proteinuria, neutropenia, hyperkalaemia, pruritic rash and taste disturbance (DiBianco, 1985). When administered during pregnancy, ACE inhibitors have potentially harmful effects to the foetus.

Table 5. Comparison of the potencies of specific competitive inhibitors against ACE of spontaneously hypertensive rats (SHR) (Cushman *et al.*, 1989).

Inhibitor	50% inhibitory concentration (IC_{50}) (nM)
Captopril	9.7
Enalapril	4645
Enalaprilat	2.8
Lisinopril	1.4
Ramipril	616
Ramiprilat	0.7

2.3.2. ACE inhibitory peptides derived from food proteins

The discovery of ACE inhibitory peptides in snake venom indicated the presence of inhibiting sequences in natural proteins. This was soon confirmed when ACE inhibitory peptides were isolated from a collagenase hydrolysate of gelatine and a trypsin hydrolysate of casein (Maruyama and Suzuki, 1982; Oshima *et al.*, 1979). Since then, numerous ACE inhibitory peptides have been isolated from food proteins, and more specifically from milk proteins (Ariyoshi, 1993; Dziuba *et al.*, 1999a; Yamamoto, 1997). A lot of ACE inhibitory peptides are derived from caseins and several of these casokinins are known to exert a significant antihypertensive effect after oral administration in SHR (Table 6). In some cases, the isolated peptides do not contribute substantially to a blood pressure lowering effect, although the hydrolysate they originate from does. For example, the peptide fraction from 8 month-aged Gouda cheese depressed the systolic blood pressure in SHR by 25 mm Hg, while two peptides isolated from this mixture, Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln and Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn, showed only a weak antihypertensive effect (Saito *et al.*, 2000). A similar observation was made by (Gobbetti *et al.*, 2000), who report that the IC_{50} of the crude peptide mixtures from fermented milk are very low, in contrast to the IC_{50} of the individual synthesised peptides. A synergistic inhibitory effect of the peptide mixtures compared to the activity of individual peptides is presumed. Whey is less extensively investigated and only few studies on antihypertensive effects of lactokinins have been reported (Table 7). Table 8 lists some ACE inhibitory peptides derived from other protein sources than milk.

Table 6. ACE inhibitory peptides derived from bovine casein (CN): casokinins.

Identified peptides	Sequence *chemical synthesis* (fragment released by human protease)	IC_{50} (µM)	Decrease in SBP (mm Hg at [mg/kg BW])°	Reference
α_{s2}-CN f(198-202)	TKVIP	400	9 [1]	Maeno *et al.*, 1996
α_{s2}-CN f(189-192)	AMKPW	580	5 [1]	
α_{s2}-CN f(190-197)	MKPWIQPK	300	3 [1]	
α_{s2}-CN f(206-207)	*YL*	122		Mullally *et al.*, 1996
α_{s2}-CN f(25-32)	NMAINPSK	60		Tauzin *et al.*, 2002
α_{s2}-CN f(81-91)	(ALNEINQFY)QK	264 (219)		
α_{s2}-CN f(92-98)	FPQYLQY	14		
α_{s2}-CN f(174-181)	(FALPQY)LK	4 (4)		
α_{s2}-CN f(182-184)	TVY	15		
α_{s1}-CN f(23-34)	(FFVAP)FPEVFGK	77 (6)	34 [100]	Karaki *et al.*, 1990; Maruyama *et al.*, 1985
α_{s1}-CN f(24-27)	*FVAP*	10		
α_{s1}-CN f(25-27)	*VAP*	2		
α_{s1}-CN f(28-34)	FPEVFGK	140		Maruyama *et al.*, 1987b
α_{s1}-CN f(32-34)	*FGK*	160		
α_{s1}-CN f(194-199)	TTM(PLW)	16 (36)	14 [100]	Karaki *et al.*, 1990; Maruyama *et al.*, 1987a
α_{s1}-CN f(197-199)	*LW*	50		Maruyama *et al.*, 1987b
α_{s1}-CN f(143-148)	AYFYPE	106		Yamamoto *et al.*, 1994b
α_{s1}-CN f(170-199)	QTQYTDAPSFSDIPN PIGSENSGKTTMPLW	346		
α_{s1}-CN f(104-109)	YKVPQL	22	13 [1]	Maeno *et al.*, 1996
α_{s1}-CN f(157-164)	DAYPSGAW	98		Pihlanto-Leppällä *et al.*, 1998
α_{s1}-CN f(194-199)	TTMPLW	51		
α_{s1}-CN f(142-147)	LAYFYP	65		
α_{s1}-CN f(1-9)	RPKHPIKHQ	13	9 [7.5]	Saito *et al.*, 2000
β-CN f(177-183)	AVPYPQR	15/274	10 [100]/ND	Karaki *et al.*, 1990; Maruyama *et al.*, 1985 / Pihlanto-Leppällä *et al.*, 1998
β-CN f(177-179)	*AVP*	340		Maruyama *et al.*, 1987b
β-CN f(177-181)	*AVPYP*	80		
β-CN f(179-181)	*PYP*	220		
β-CN f(43-68)	DELQDKIHPFAQTQSI VYPFPGPIPNS	4		Yamamoto *et al.*, 1994b
β-CN f(191-202)	LLYQQPVLGPVRGPF PIIV	21	22* [15]	
β-CN f(158-175)	PPQSVLSLSQSKVLP VPE	25		
β-CN f(168-175)	SKVLPVPE	39		
β-CN f(60-66)	YPFPGPI	500		Meisel and Schlimme, 1994
β-CN f(193-202)	YQQPVLGPVR	300		
β-CN f(74-76) κ-CN f(108-110)	IPP	5	24 [0.3]	Nakamura *et al.*, 1995a; Nakamura *et al.*, 1995b
β-CN f(84-86)	VPP	9	29 [0.6]	

°Maximal decrease in Systolic Blood Pressure (SBP) after oral administration in Spontaneously Hypertensive Rats (SHR).

*A hydrolysate of β-CN containing these peptides exerted the antihypertensive effect.

Table 6. ACE inhibitory peptides derived from bovine casein (CN): casokinins (continued).

Identified peptides	Sequence *chemical synthesis* (fragment released by human protease)	IC_{50} (μM) (μg/ml)[§]	Decrease in SBP (mm Hg at [mg/kg BW])°	Reference
β-CN f(169-175)	(KVLPVP)Q	1000 (5)	24 [1] (32 [1])	Maeno *et al.*, 1996
β-CN f(140-143)	LQSW	500	2 [1]	
β-CN f(59-64)	VYPFPG	221	22 [8]	Abubakar *et al.*, 1998
β-CN f(59-61)	VYP	288	21 [8]	
β-CN f(80-90)	TPVVVPPFLQP	749	8 [8]	
β-CN f(58-72)	LVYPFPGPIPNSLPQ	18		Smacchi and Gobbetti, 1998
β-CN f(108-113)	EMPFPK	423[§]		Pihlanto-Leppällä *et al.*, 1998
β-CN f(193-198)	YQQPVL	280		
β-CN f(60-68))	YPFPGPIPN	15	7 [7.5]	Saito *et al.*, 2000
β-CN f(6-14)	LNVPGEIVE	291		Gobbetti *et al.*, 2000
β-CN f(73-82)	NIPPLTQTPV	180		
β-CN f(47-52)	DKIHPF	194		
α_{s1}-CN f(146-147)	YP	720	27 [1]	Yamamoto *et al.*, 1999
α_{s1}-CN f(159-160)				
β-CN f(114-115)				
κ-CN f(58-59)				

°Maximal decrease in Systolic Blood Pressure (SBP) after oral administration in Spontaneously Hypertensive Rats (SHR).

ACE inhibitory peptides display a lower ACE inhibitory activity *in vitro* than the ACE inhibitory drugs, yet do not have the harmful side effects associated with synthetically produced drugs (Fitzgerald and Meisel, 2000). Due to the physiological role of ACE in the kallikrein-kinin system, ACE inhibitory peptides may also possess immunomodulatory properties (Meisel, 1998).

As for the ACE inhibitory drugs, structure-activity correlations between different peptide inhibitors of ACE indicate that binding to ACE is strongly influenced by the C-terminal tripeptide sequence of the substrate. Although the precise substrate specificity is not fully understood, ACE appears to prefer substrates or competitive inhibitors containing hydrophobic (aromatic or branched side-chains) amino acid residues at the three C-terminal positions. However, a C-terminal lysine or arginine, with a positive charge on the ε-amino group, seems also to contribute substantially to the inhibitory potency. In this respect, it is postulated that the mechanism of ACE inhibition involves inhibitor interaction with an anionic binding site which is distinct from the catalytic site. Therefore, it is expected that peptide conformation, i.e. the structure adopted in a specific environment, should contribute to ACE inhibitor potency. Due to substrate specificity differences between the two catalytic sites of ACE, ACE inhibitors may inhibit only one catalytic site.

Table 7. ACE inhibitory peptides derived from bovine whey: lactokinins.

Identified peptides	Sequence *chemical synthesis* (fragment released by human protease)	IC_{50} (µM)	Decrease in SBP (mm Hg at [mg/kg BW])°	Reference
α-LA f(50-53)	*YGLF* α-lactorphin*	733	23 [0.1]	Mullally *et al.*, 1996; Nurminen *et al.*, 2000
α-LA f(105-110)	LAHKAL	621		Pihlanto-Leppällä *et al.*, 1998
α-LA f(50-52)	YGL	409		Pihlanto-Leppälä *et al.*, 2000
α-LA f(99-108)	VGINYWLAHK	327		
α-LA f(104-108)	WLAHK	77		
α-LA f(52-53)	*LF*	349		Mullally *et al.*, 1996
β-LG f(104-105)				
β-LG f(102-103)	*YL*	122		
β-LG f(102-105)	*YLLF* β-lactorphin*	172		
β-LG f(146-149)	*HIRL* β-lactotensin	1153		
β-LG f(147-148)	*IR*	696		
β-LG f(142-148)	ALPMHIR	43		Mullally *et al.*, 1997b
β-LG f(146-148)	*HIR*	954		
β-LG f(9-14)	GLDIQK	580		Pihlanto-Leppällä *et al.*, 1998
β-LG f(78-80)	IPA	141	31 [8]	Abubakar *et al.*, 1998
β-LG f(81-83)	VFK	1029		Pihlanto-Leppälä *et al.*, 2000
β-LG f(22-25)	LAMA	556		
β-LG f(32-40)	LDAQSAPLR	635		
β-LG f(106-111)	CMENSA	788		
β-LG f(142-146)	ALPMH	521		
β-LG f(94-100)	VLDTDYK	946		
BSA f(208-216)	*ALKAWSVAR*	3		Chiba and Yoshikawa, 1991
BSA f(221-222)	FP	315	27 [8]	Abubakar *et al.*, 1998

°Maximal decrease in Systolic Blood Pressure (SBP) after oral administration in Spontaneously Hypertensive Rats (SHR).

*α-lactorphin can be produced by proteolysis of α-LA with pepsin alone or in combination with trypsin, while β-lactorphin can be released from β-LG by pepsin and trypsin (and chymotrypsin) (Antila *et al.*, 1991).

A detailed knowledge of the mechanism of action of ACE and the conformational behaviour of ACE inhibitory peptides should lead to a better understanding of the antihypertensive potential of milk derived peptides (Fitzgerald and Meisel, 2000).

As an example of a structure-activity relationship, the ACE inhibitory activity of dipeptides with tyrosine is higher than those with phenylalanine, but less than dipeptides with proline at the C-terminal (Cheung *et al.*, 1980). After oral administration in SHR, dipeptides with tyrosine at the C-terminal caused slow but prolonged reduction of the systolic blood pressure (SBP) compared to dipeptides with phenylalanine at the carboxy-terminus, which produced a more rapid decrease and a shorter duration of action (Suetsuna, 1998).

Table 8. Some ACE inhibitory peptides derived from other protein sources than milk.

Protein source	Sequence *chemical synthesis* (fragment released by human protease)	IC_{50} (µM)	Decrease in SBP (mm Hg at [mg/kg BW])°	Reference
gelatin	GPAGAZ + GPPGAZ	8		Oshima *et al.*, 1979
α-zein	LRP	0.3	15 [30][a]	Miyoshi *et al.*, 1991a
porcine muscle	MNP	67		Arihara *et al.*, 2001
human plasma	LIY acein-2	0.8		Nakagomi *et al.*, 2000
chickpea		0.1		Pedroche *et al.*, 2002
wakame	YNKL	21	50 [50]	Suetsuna and Nakano, 2000
garlic	FY	4	25 [200]	Suetsuna, 1998
buckwheat	YQY	4	30 [100][b]	Li *et al.*, 2002
royal jelly	DGL	2	23 [1000]	Matsui *et al.*, 2002c
wheat germ	I(VY)	0.5 (5)	19 [5][a] (18 [50])	Matsui *et al.*, 1999; Matsui *et al.*, 2000
dried bonito	(LKP)NM	2 (0.3)	14 [15] (16 [9])	Fujita and Yoshikawa, 1999
swine hemoglobin	FQKVVA	6	30 [50]	Mito *et al.*, 1996
bonito bowel	IRPVQ	1	19 [100]	Karaki *et al.*, 1993
soy	DLP	5	38 [100][b]*	Wu and Ding, 2001; Wu and Ding, 2002

°Maximal decrease in Systolic Blood Pressure (SBP) after oral administration in Spontaneously Hypertensive Rats (SHR).
[a]Intravenous injection of the peptide in SHR.
[b]A hydrolysate containing this peptide exerted the antihypertensive effect.
*Oral administration during 1 month.

2.3.3. Determination of the ACE inhibitory activity and the antihypertensive effect

For measuring the ACE inhibitory activity *in vitro*, pure ACE or a concentrated lung extract from rabbit, pig, rat or dog is applied as enzyme source. Determination of the ACE inhibitory activity implies the determination of the ACE activity. The first methods reported for the ACE assay utilised the physiological substrates, but suffered from interference by other peptidases that degrade both substrate and products. Based on the physical, chemical and enzymatic qualities of ACE, several synthetic substrates with amino-substituted tri- and dipeptides were developed that allow a sensitive and direct measurement of the ACE activity via radioisotopic, spectrophotometric, fluorometric and chromatographic methods. From the ACE activity in the absence and the presence of an inhibitor, the percent ACE inhibition can be deduced. When this is done for different concentrations of inhibitor, the IC_{50} value, which is the concentration of inhibitor needed to reduce the ACE activity to half of its initial value, can be calculated. The ACE inhibitory activity is usually expressed as IC_{50} value.

The most applied substrate is hippuryl-L-histidyl-L-leucine (HHL). By the action of ACE, HHL is degraded to hippuric acid and His-Leu (Figure 7). In the original method of Cushman and Cheung (1971), rabbit lung acetone powder extract serves as ACE source and the rate

of production of the extracted hippuric acid, a direct measure for the ACE activity, is determined spectrophotometrically. An extraction step is necessary, as both hippuric acid and HHL absorb light of similar wavelength.

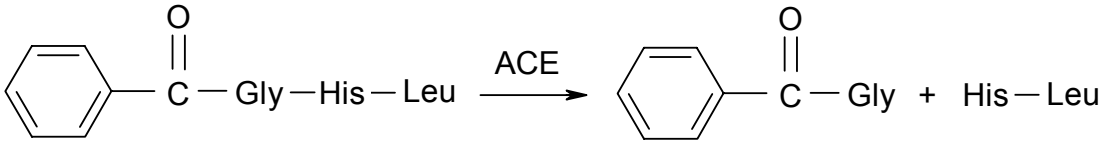


Figure 7. ACE catalysed reaction in the method of Cushman and Cheung (1971) with substrate HHL.

Later on, some modifications to this method were made by Nakamura *et al.* (1995a), who used pure ACE from rabbit lung. Some compounds in the inhibitor sample may be extracted together with hippuric acid into ethyl acetate and consequently interfere with the UV determination. Therefore, ACE inhibitory activity assays are described based on RP-HPLC separation of hippuric acid, HHL and other interfering compounds (Kim *et al.*, 1999; Mehanna and Dowling, 1999). The substrate HHL may also be used to measure ACE activity fluorimetrically by formation of the fluorescent adduct of o-phtaldialdehyde and the histidyl moiety of the product His-Leu (Friedland and Silverstein, 1976). Another variant is based on color development by specific binding of 2,4,6-trinitrobenzene sulfonate (TNBS) to the primary amine of His-Leu (Matsui *et al.*, 1992). The latter two methods have the disadvantage that fluorescent and colour adducts may be formed with other peptides and that His-Leu may be cleaved by proteases, present in the inhibitor sample. An extra blank containing all assay components, but where the reaction of ACE was prevented by acid or base addition, should correct for this.

Another substrate for ACE is 2-furanacryloyl-L-phenylalanyl-L-glycyl-L-glycine (FAPGG). By the action of ACE, the dipeptide Gly-Gly is formed and a blue shift of the absorption spectrum occurs (Figure 8).

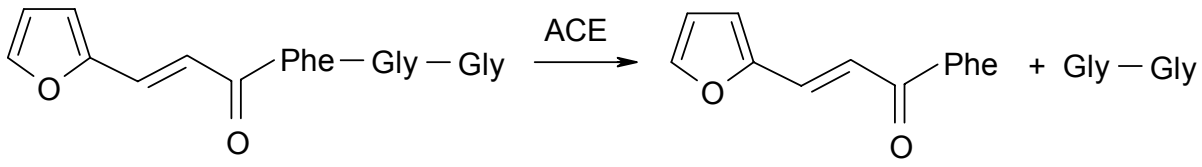


Figure 8. ACE catalysed reaction in the method of Holmquist *et al.* (1979) with substrate FAPGG.

In the original method of Holmquist *et al.* (1979), pure ACE from rabbit lung acetone powder serves as enzyme source and the decrease in absorbance at 330-350 nm during 1 to

10 min is a measure for the ACE activity both at high and low substrate concentrations. ACE exhibits high affinity for FAPGG and the kinetic parameters of its hydrolysis are better than for HHL. This method was slightly modified by some authors (Ronca-Testoni, 1983; Wong and Kinniburgh, 1987).

The chromophore- and fluorophore-labelled tripeptide dansyltriglycine can also be used as substrate and is cleaved by pure ACE from rabbit lung into dansylglycine and Gly-Gly. The product dansylglycine and unreacted substrate are separated and quantified by RP-HPLC (Elbl and Wagner, 1991). This method is well suitable for the screening of crude plant extracts, where some constituents may interfere with spectrophotometric or fluorometric determination.

As the substrate specificity of ACE is very broad, many ACE substrates can be present in processed food proteins. In any ACE inhibition assay, these substrates show seemingly ACE inhibitory activity *in vitro*, but they fail to produce an antihypertensive effect *in vivo*. Pre-incubation of the pure peptide with ACE before the measurement of the ACE inhibitory activity discriminates substrates from true inhibitors. In the case of an ACE substrate, the IC_{50} value will increase after pre-incubation with ACE (Fujita *et al.*, 2000).

Comparison of IC_{50} values in literature is hampered by the use of different substrates, different sources of ACE, different determination methods, different assay conditions and different ways of calculation (Ariyoshi, 1993; Brooks *et al.*, 1990; Buttery, 1985; Nakamura *et al.*, 1995a; Weisser and Schloos, 1991). Even when using the same method, different IC_{50} values have been reported, like for Ala-Val-Pro-Tyr-Pro-Gln-Arg in Table 6.

While the ACE inhibitory activity is a marker for a biological response, the demonstration of an antihypertensive effect represents an intermediate endpoint marker for cardiovascular disease. Antihypertensive effects are usually measured in spontaneously hypertensive rats (SHR), which are genetically predisposed to have a high blood pressure (Yigal *et al.*, 1998). The change in blood pressure is monitored in conscious and anesthetised rats by the tail-cuff method (Widdop and Li, 1997) or via a catheter in the artery, after administration of the product orally, intravenously or intraperitoneally (Fujita and Yoshikawa, 1999; Miyoshi *et al.*, 1991b). Normally ACE inhibitory peptides only produce an antihypertensive effect in SHR, while no effect is observed in normotensive or Wistar Kyoto (WKY) rats. Previously, ACE inhibitory peptides were administered intravenously to normal rats and the antagonism to an elevated blood pressure response after intravenous injection of angiotensin I was measured *in vivo* (Maruyama *et al.*, 1989). ACE inhibitory peptides have also been tested on stroke-prone spontaneously hypertensive rats, which are strongly predisposed to hypertension and stroke (Mizutani *et al.*, 2000).

2.4. Hypertension

Hypertension is defined as a sustained increase in blood pressure, more specifically a systolic blood pressure of 140 mm Hg or more and/or a diastolic blood pressure of 90 mm Hg or more. Hypertension is one of the most common chronic medical conditions in the developed world and is rapidly becoming a major problem in developing countries. It is estimated that about 20% of the world's adult population suffers from hypertension. The prevalence of high blood pressure increases with age, affecting approximately 65% of the population aged 65-74 years in Western nations (Alper *et al.*, 2001; Duprez *et al.*, 2002).

High blood pressure is a haemodynamic disorder in which increased arterial pressure may be associated with an increased cardiac output or increased peripheral resistance. Most cases of hypertension result from the interplay of genetic and environmental factors, with 25-40% of blood pressure variation being genetically determined. Hypertension has clearly been demonstrated to be a leading risk factor for stroke, coronary heart disease, heart failure and renal failure. As there are often no clinical manifestations other than increased blood pressure, hypertension is also named the 'silent killer'.

Lifestyle modifications are recommended in the prevention, but also in the treatment of hypertension. Not only do these measures help to lower the blood pressure, they also help to reduce the need for pharmacological treatment. In this respect, it is advised to lose weight if overweight, to limit alcohol intake to no more than one or two drinks per day, to increase aerobic physical activity, to reduce sodium intake, to increase the intake of fresh fruits and vegetables, which should increase the intake of potassium, calcium and vitamins, and to stop smoking. Several recent clinical trials have demonstrated the benefits of dietary modification in blood pressure control (Groziak and Miller, 2000; Hermansen, 2000). Hypertensive subjects enrolled in the Dietary Approaches to Stop Hypertension trial (DASH), a diet rich in fruits, vegetables and low-fat dairy products, were shown to have a blood pressure reduction of 11.4/5.5 mm Hg, compared with a normal American diet and despite dietary sodium and subject weight being held constant.

The goal of antihypertensive therapy is to reduce cardiovascular morbidity and mortality and to prolong life by the least intrusive means possible. Seven different classes of antihypertensive drugs are currently in use:

(1) diuretics, which cause the body to excrete water and salt.
(2) β-blockers and α-blockers, which block the effect of adrenaline, thus easing the heart's pumping action and widening blood vessels.
(3) ACE inhibitors, which reduce the production of the vasoconstrictor angiotensin II.

(4) calcium channel antagonists, which help decrease the contractions of the heart and widen blood vessels.
(5) angiotensin II receptor blockers, which block the effect of the vasoconstrictor angiotensin II, have similar benefits as ACE inhibitors and may have fewer or less severe side effects, because they do not interfere in the kallikrein-kinin system.
(6) vasodilators, which widen blood vessels.

It is important to ensure continuous administration of the antihypertensive drugs. As first-line treatment, experts generally recommend β-blockers or diuretics, which are inexpensive, safe, and effective for most people with hypertension who have no complicating problems. Individuals, however, may have special requirements that call for specific drugs or combinations (Alper *et al.*, 2001). Combining low doses of drugs with different mechanisms of action, is suggested to result in an additive or synergistic effect on blood pressure (Messerli, 1999; Taylor, 2001).

The role of ACE in the pathology of hypertension remains obscure. However, in human subjects an ACE gene insertion/deletion (I/D) polymorphism has been observed. This insertion consists of a 287-bp-long *alu* repetitive sequence located inside intron 16 of the ACE gene. In subjects who are homozygous for the D allele, the mean serum ACE is nearly twice as high as that of subjects with the II genotype (Soubrier *et al.*, 1993b). Furthermore, the DD genotype is associated with an increased risk for cardiovascular diseases (Soubrier *et al.*, 1993b), and even cancer and psychiatric disease (Moskowitz, 2002).

2.5. The role of peptides in the treatment of cardiovascular diseases

Food derived peptides are known to lower the blood pressure by other mechanisms than ACE inhibiton.

The opiate and ACE inhibitory peptide α-lactorphin (Table 7), originally derived from bovine α-lactalbumin, dose-dependently lowers the blood pressure of both SHR and normotensive Wistar Kyoto rats (WKY) upon subcutaneous administration. The reduction in blood pressure can be reversed by nalaxone, a specific opiate receptor antagonist, providing evidence for the involvement of opiate receptors in the antihypertensive effect (Nurminen *et al.*, 2000).

Single oral administration of an extract from autologous lysate of *Lactobacillus casei* significantly lowers the blood pressure in spontaneously hypertensive rats and long-term oral administration even suppresses the development of hypertension in these animals (Furushiro *et al.*, 1990). The active substance of this extract, a polysaccharide-glycopeptide complex, enhances only after oral intake prostaglandin synthesis, which resulted in a decrease of

peripheral vascular resistance and hence an antihypertensive effect (Furushiro *et al.*, 1993). Furthermore, oral administration of the *Lactobacillus casei* cell lysate extract to hypertensive patients reduces both the systolic and diastolic blood pressure, in addition to the total cholesterol and fasting plasma glucose level (Nakajima *et al.*, 1995).

Two peptides, the ovokinin Phe-Arg-Ala-Asp-His-Pro-Phe-Leu and Arg-Ala-Asp-His-Pro-Phe, isolated from a pepsin and α-chymotrypsin digest of ovalbumin respectively, exert a dose-dependent vasodilatation in an isolated mesenteric artery in SHR, which is preconstricted by phenylephrine. The vasorelaxing activity is attributable to the binding to B_1 receptors and the subsequent release of prostaglandin and NO respectively. Following oral administration, Arg-Ala-Asp-His-Pro-Phe lowers the blood pressure in SHR (Fujita *et al.*, 1995; Matoba *et al.*, 1999). An even more potent and long-lasting hypotensive activity is obtained by oral administration of the synthetic analogue Arg-Pro-Phe-His-Pro-Phe (Matoba *et al.*, 2001).

Pepsin digests of bonito and beef inhibit the endothelin converting enzyme, which produces the potent vasoconstrictor endothelin (Okitsu *et al.*, 1995).

3. PRODUCTION OF ACE INHIBITORY PEPTIDES

3.1. Introduction

Sometimes, ACE inhibitory peptides are present as such in foods, for example in garlic (Suetsuna, 1998) and the mushroom *Grifola frondosa* (Choi *et al.*, 2001). However, most of the time, they need to be specifically released from the food protein by the action of enzymes, heat, base or acid. Hence, ACE inhibitory peptides are usually formed during food processing or during gastrointestinal digestion in the human body.

3.2. Fermentation

3.2.1. Introduction

Several ACE inhibitory peptides have been isolated from fermented products, mostly milk derived products. The proteolytic system of lactic acid bacteria can contribute to the liberation of bioactive peptides (Law and Haandrikman, 1997). The cell wall bound proteases release oligopeptides from the milk proteins with a rather broad substrate specificity. Transport systems specific for amino acids and peptides up to 18 amino acids are present in lactic acid bacteria for nitrogen uptake. Longer oligopeptides, not transported into the cells,

can be a source of bioactive peptides in fermented milk products when further degraded, for example by the intracellular peptidases of lactic acid bacteria after cell lysis. As casein contains a lot of proline, especially the peptidases associated with the hydrolysis of proline-containing peptides have received considerable attention: aminopeptidase P, prolidase, proline iminopeptidase, prolinase and X-prolyl-dipeptidyl aminopeptidase. However, the specificities of the known peptidases suggest that almost all peptide bonds in caseins can be cleaved in theory (Christensen *et al.*, 1999; Kunji *et al.*, 1996). In this respect, the formation of bioactive peptides by lactic acid bacteria in fermented milk products seems to be a rare event. Nevertheless, bioactive peptides are isolated from fermented milk products, suggesting limited proteolysis conditions and/or resistance towards proteolysis (Meisel and Bockelmann, 1999).

3.2.2. The success story of Lactobacillus helveticus and Calpis™ milk

The lactic acid bacterium *Lactobacillus helveticus* is particularly well-known for the production of ACE inhibitory peptides. This microorganism possesses efficient protease and peptidase activities towards milk proteins (Matar *et al.*, 1996). Compared to other lactic acid bacteria, milk ferments obtained from most strains of *L. helveticus* show high ACE inhibitory activity and a significant blood pressure lowering effect. Furthermore, the fermented milks with higher ACE inhibitory activity show a better growth of the lactic acid bacterium associated with a larger pH decrease, a higher peptide content and a higher protease activity (Yamamoto *et al.*, 1994a).

Milk fermented for 24 h at 37°C with the Calpis™ starter, including *L. helveticus* and *Saccharomyces cerevisiae*, contains the highly potent ACE inhibitory peptides Val-Pro-Pro and Ile-Pro-Pro derived from β-casein and κ-casein (Table 6) (Nakamura *et al.*, 1995a). It seems that these peptides are processed by the extracellular protease of *Lactobacillus helveticus* followed by some peptidase action during fermentation (Yamamoto *et al.*, 1993; Yamamoto *et al.*, 1994b). Both the sour Calpis™ milk and the isolated peptides demonstrate an antihypertensive effect in SHR of at least 20 mm Hg from 4 to 8 h after a single oral administration of 5 ml/kg BW for the Calpis™ milk, equalling 0.6 mg Val-Pro-Pro/kg BW and 0.3 mg Ile-Pro-Pro/kg BW, and 5 mg/kg BW for Val-Pro-Pro and Ile-Pro-Pro respectively. Moreover, the antihypertensive effect of the peptides is dose-dependent. In contrast, a single oral administration of either sour milk or tripeptides does not change the systolic blood pressure of normotensive WKY rats (Nakamura *et al.*, 1995b). Val-Pro-Pro and Ile-Pro-Pro are also detected in the abdominal aorta of SHR 6 h after oral administration of the sour milk. The ACE inhibitory activity in the aorta is lower in rats fed with the sour milk compared to control rats (Masuda *et al.*, 1996). This clearly indicates that the peptides resist

gastrointestinal digestion, are absorbed intact into the blood and exert an antihypertensive effect by ACE inhibition.

In a placebo-controlled study, hypertensive patients ingest daily 95 ml of the sour milk or a placebo for 8 weeks, in addition to their antihypertensive medication. Both the systolic and diastolic blood pressure decrease significantly in the treatment group and these decreases are maintained 4 weeks after treatment (Figure 9). The dose of sour milk is approximately 2 ml/kg BW, which corresponds to a dose of 0.033 mg Val-Pro-Pro and 0.025 mg Ile-Pro-Pro per kg BW (Hata *et al.*, 1996). In a similar study, these results are confirmed in untreated hypertensive subjects and no effect is observed in healthy individuals with normal blood pressure (Itakura *et al.*, 2001).

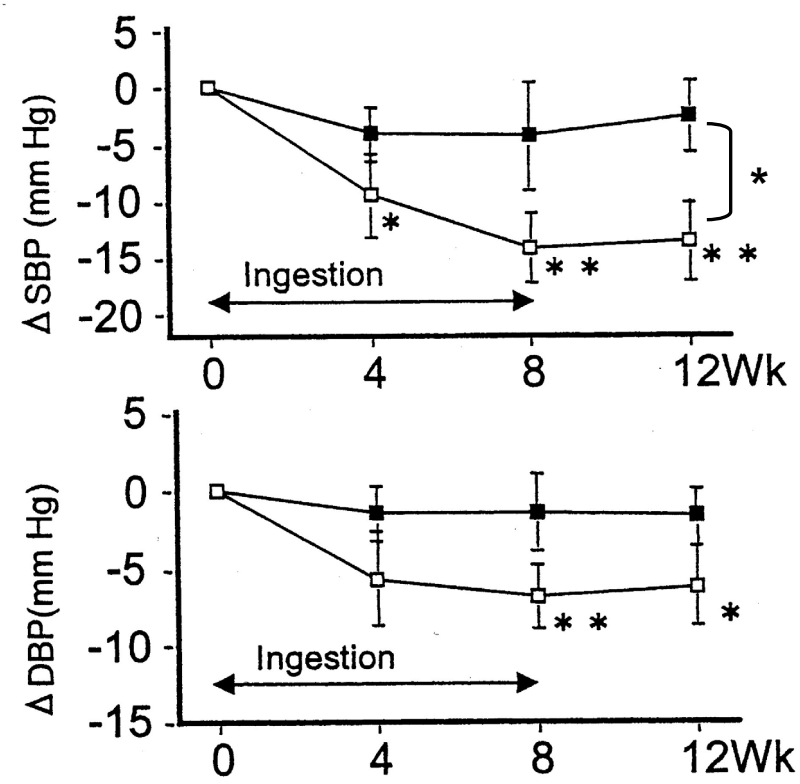


Figure 9. Changes in systolic blood pressure (SBP) and diastolic blood pressure (DBP) of hypertensive subjects by treatment for 8 weeks with Calpis™ sour milk (white) and artificially acidified milk as placebo (black). Significant difference from initial value: *p < 0.05, **p < 0.01 (Takano, 2002).

In addition to the beneficial effects of Calpis™ milk in the treatment of hypertension, it may also have a function in the prevention of hypertension (Takano, 1998). In this respect, in young SHR fed with the Calpis™ milk for 15 weeks, the ACE inhibitory activity of the aorta is lower and the systolic blood pressure increase is inhibited compared to rats fed a normal diet

(Nakamura *et al.*, 1996). Based on all this evidence, a functional food product containing sour milk as a significant component is approved as “Food for Specified Use” in Japan with an appropriate health claim on the label (Takano, 2002).

In a yoghurt-like product fermented by *L. helveticus* CPN4 for 10 h, the casein-derived peptide Tyr-Pro yields significant antihypertensive activity in SHR (Table 6). As this sequence is found at more C-terminal regions of bovine caseins and the protease of this strain preferentially hydrolyses casein at the C-terminus, it is suggested that this peptide is likely to be formed at the beginning of fermentation. As this product does not contain Val-Pro-Pro and Ile-Pro-Pro, a longer fermentation time would be necessary to release these peptides (Yamamoto *et al.*, 1999).

Other research groups have also used *L. helveticus* to produce fermented milk with antihypertensive properties (Fuglsang *et al.*, 2002; Leclerc *et al.*, 2002; Seppo *et al.*, 2002; Seppo *et al.*, 2003). In a long-term study, the development of hypertension in SHR is significantly and dose-dependently attenuated when Calpis™ milk or Evolus® milk, containing the double dose of Val-Pro-Pro and Ile-Pro-Pro, or the tripeptides itself, are administered daily (Sipola *et al.*, 2001; Sipola *et al.*, 2002).

3.2.3. Other fermented products

High ACE inhibitory activities are obtained in fermented milks started by *Lactobacillus delbrueckii* ssp. *bulgaricus* SS1 and *Lactococcus lactis* ssp. *cremoris* FT4, respectively. The IC_{50} of the crude peptide fractions are very low, 8-11 mg/l, while the isolated peptides display a considerable lower ACE inhibitory activity (Gobbetti *et al.*, 2000). When milk whey and casein protein are fermented by different lactic acid starters on the other hand, digestion with pepsin and trypsin is necessary to observe ACE inhibitory activity. The starters were *Streptococcus salivarius* ssp. *thermophilus*, bifidobacteria and *Lactobacillus acidophilus* for the yoghurt; *Lactococcus lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris*, *L. lactis* ssp. *lactis* biovar. *diacetylactis* and *Leuconostoc mesenteroides* spp. c*remoris* for the ropy milk; and *L. lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris*, *L. lactis* ssp. *diacetylactis* and *Leuconostoc mesenteroides* spp. *cremoris* for the soured milk. These results may be due to the low proteolytic activity of the used starters or the specificity of the enzymes in the lactic acid bacteria (Pihlanto-Leppällä *et al.*, 1998). Aged fermented milk prepared by culturing with various lactic acid bacteria and yeast exhibits a significant antihypertensive effect in SHR following oral intake, despite its weak ACE inhibitory activity *in vitro* (Kuwabara *et al.*, 1995). In fed-batch fermentation by *Kluyveromyces marxianus* var. *marxianus* of whey protein, several peptides are isolated that contain the sequences of known ACE inhibitory peptides (Belem *et al.*,

1999). These peptides may be released upon oral administration and lower the blood pressure.

ACE inhibitory peptides have been isolated from several natural and modified cheeses (Haileselassie *et al.*, 1999; Ryhänen *et al.*, 2001; Smacchi and Gobbetti, 1998). Upon assessment of different milk products, ACE inhibitory activity is especially observed in ripened cheeses. In these products, the ACE inhibitory activity increases during cheese maturation, but decreases when the proteolysis exceeds a certain level (Figure 10) (Meisel *et al.*, 1997). In agreement with this observation, the free peptides from 8 month-aged Gouda cheese exert the strongest depressive effect in the systolic blood pressure after oral administration in SHR, compared to 24 month-aged Gouda, Emmental, Blue, Camembert, Edam and Havarti cheeses (Saito *et al.*, 2000).

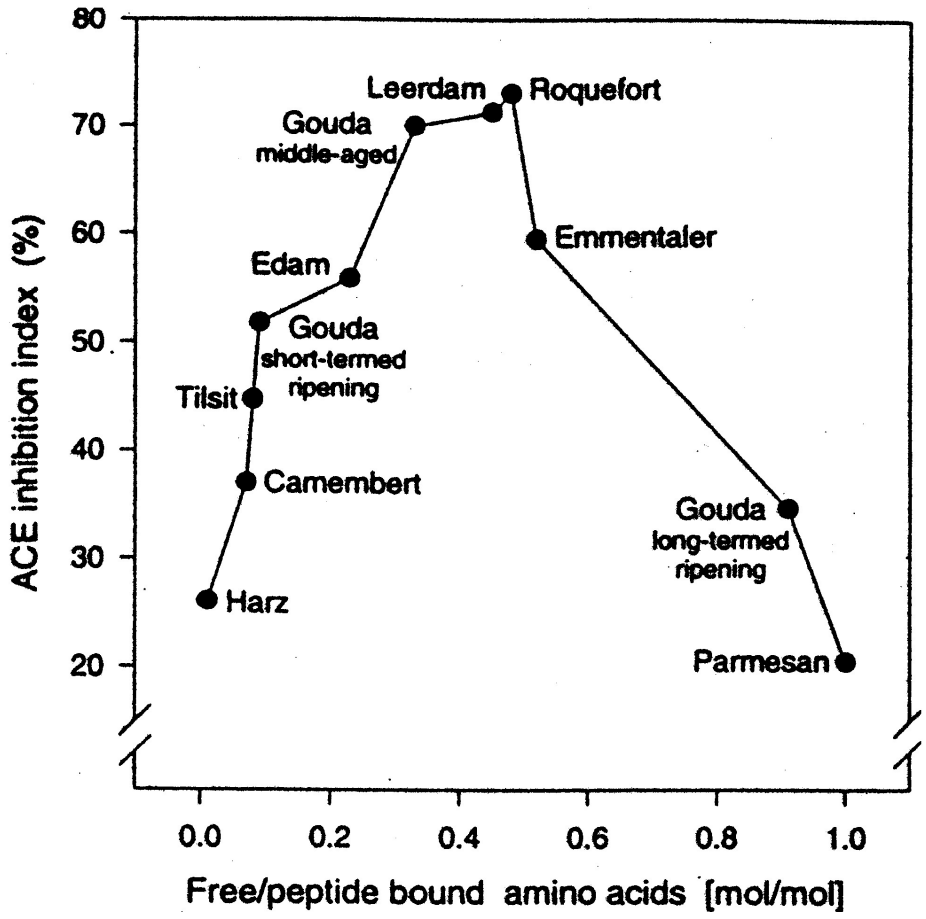


Figure 10. ACE inhibition index (%) of extracts from ripened cheese in dependence on proteolysis as measured by the ratio of free to peptide-bound amino acids (Meisel *et al.*, 1997).

The ACE inhibitory peptide His-His-Leu (IC_{50} = 5 µM) is present in Korean fermented soybean paste and triple intravenous injections of this peptide in SHR at a total dose of 5 mg/kg BW results in a significant decrease of the systolic blood pressure by 61 mmHg (Shin *et al.*, 2001). Potent ACE inhibitory peptides are also isolated from sake and sake lees. An antihypertensive effect is observed with Ile-Tyr-Pro-Arg-Tyr at 100 mg/kg BW in SHR that continues for 30 h after oral administration. This peptide maintains its inhibitory activity when digested with pepsin and pancreatin *in vitro* and three dipeptide fragments of this pentapeptide, which may be released by brush border and plasma peptidases, have hypotensive effects in SHR as well (Saito *et al.*, 1994). Furthermore, ACE inhibitory peptides with IC_{50} of 17.5-83 µM are found in red wine (Takayanagi and Yokotsuka, 1999).

3.3. Digestion

3.3.1. Introduction

Digestion of proteins is usually performed by microbial enzymes or gastrointestinal proteases. ACE inhibitory peptides may be released by one enzyme or by a combination of enzymes in batch or in a continuous process (Table 9).

3.3.2. Fermentation related enzymes

When the research group of the Calpis™ milk hydrolyses β-casein with an extracellular protease from *L. helveticus* CP790, they cannot isolate Val-Pro-Pro or Ile-Pro-Pro, but find the antihypertensive peptide Lys-Val-Leu-Pro-Val-Pro-Gln (Table 6) (Maeno *et al.*, 1996). This also demonstrates that a longer incubation time and/or other peptidase action is required to release these tripeptides. Maybe they are produced by peptidases of *Saccharomyces cerevisiae*. This yeast has been shown to contain several proteolytic enzymes, such as protease A, protease B, carboxypeptidase Y, carboxypeptidase S, aminopeptidase Co and dipeptidyl aminopeptidase B (Jones, 1991). Potent ACE inhibitory activity is identified in skimmed milk digested with cell-free extract of *S. cerevisae* and the active enzyme is characterised as protease B (Roy *et al.*, 2000).

3.3.3. Comparison of different proteases

Often different enzymes are compared for their ability to release ACE inhibitory peptides from a certain food protein. When trypsin, α-chymotrypsin, proteinase K and thermolysin hydrolyse ovine and caprine β-lactoglobulin for 24 h, the highest ACE inhibitory activity is obtained with the microbial enzymes proteinase K and thermolysin. Apparently, these proteases yield peptides of lower molecular masses than the digestive enzymes.

Table 9. Some proteases used in the production of ACE inhibitory peptides (less specific cleavage).

Protease	Origin	Specificity
Gastrointestinal:		
pepsin (A or C)	porcine stomach	(C-) and N-terminus of Phe, Leu, Tyr, (Trp)
trypsin	bovine pancreas	C-terminus of Arg, Lys
α-chymotrypsin	bovine pancreas	C-terminus of Trp, Tyr, Phe, (Leu, Met, His)
elastase	bovine pancreas	C-terminus of uncharged, non-aromatic amino acids: Ala, Val, Leu, Ile, Gly
carboxypeptidase A	bovine pancreas	cleaves C-terminal aromatic or aliphatic (neutral) amino acids
carboxypeptidase B	bovine pancreas	cleaves C-terminal basic amino acids
pancreatin	porcine/bovine pancreas	endo- and exopeptidases
Others:		
thermolysin	*Bacillus thermoproteolyticus*	N-terminus of Leu, Phe, (Trp, Tyr, Ile, Val, Met)
proteinase K	*Tritirachium album*	C-terminus of aromatic or aliphatic (hydrophobic) amino acids
alkalase®	*Bacillus licheniformis*	endopeptidase (subtilisin A), broad, preference for C-terminus of large uncharged amino acid
neutrase®	*Bacillus subtilis*	endopeptidase
flavourzyme®	*Aspergillus oryzae*	endo- and exopeptidase (carboxy-, amino- and X-prolyl dipeptide aminopeptidase)
pronase E	*Streptomyces griseus*	broad, endoprotease
actinase E	*Actinomyces* spp.	broad, endoprotease
newlase F	*Rhizopus spp.*	broad, endoprotease
papain	*Carica papaya*	broad, endoprotease
ficin	Fig tree latex	broad, endoprotease
collagenase		N-terminus of Gly in Pro-X-Gly-Pro
protease of *L. helveticus*	*L. helveticus* CP790	broad specificity, N-terminus of Leu, Phe, Ser, Lys, Glu, and Gln

®Industrial enzymes from Novo Nordisk.

Ovine and caprine β-lactoglobulin hydrolysates have similar effects, but sweet whey results in a considerable lower IC_{50} than acid whey with the exception of the digestion by α-chymotrypsin. This points to the release of potent ACE inhibitory peptides from casein glycomacropeptide, only present in sweet whey (Hernandez-Ledesma *et al.*, 2002).

When porcine skeletal muscle protein is digested by eight different proteases, the thermolysin digest has the highest ACE inhibitory activity, followed by proteinase K, papain and pronase E. α-chymotrypsin and ficin are less effective and trypsin and pepsin produce only little ACE inhibitory activity (Arihara *et al.*, 2001).

In another study, wheat germ is digested by pepsin, α-chymotrypsin, trypsin, alkalase and protease from *Aspergillus niger* for 5 h under various pre-treatment conditions. For all pre-treatments, hydrolysis by alkalase is the most favourable in producing potent ACE inhibitory activity. A pre-treatment consisting of defatting the wheat germ followed by α-amylase digestion results in a higher ACE inhibitory activity upon digestion compared to intact wheat. By removing lipids and sugars from the matrix, the susceptibility of the wheat germ protein to proteolysis increases (Matsui *et al.*, 1999).

Alkalase is also the most effective protease to release ACE inhibitory peptides from blood plasma proteins, followed by trypsin, while neutrase, pepsin and papain are less effective. Trypsin on the other hand produces in a lower enzyme concentration higher ACE inhibitory activity from casein than alkalase (Hyun and Shin, 2000).

This indicates that the capability of an enzyme to produce ACE inhibitory peptides is dependent on the food protein sequence.

Upon oral administration of seven different whey digests to SHR, significant antihypertensive activity is observed after 6 h for the digests with trypsin, proteinase K and actinase E (Table 10) (Abubakar *et al.*, 1998). The non-digested whey protein shows already an antihypertensive effect, but no ACE inhibitory activity. This may be explained by the formation of ACE inhibitory peptides during gastrointestinal digestion and/or an antihypertensive effect by another mechanism than ACE inhibition. For the different digests, the ACE inhibitory activity does not always correspond with the antihypertensive effect.

The thermolysin-digest of 'Katsuo-bushi', a Japanese traditional food processed from dried bonito, possesses potent ACE inhibitory activity and demonstrates antihypertensive activity after oral administration in hypertensive subjects. One of the isolated peptides, Leu-Lys-Pro-Asn-Met, is hydrolysed by ACE to Leu-Lys-Pro, a very potent ACE inhibitory and antihypertensive peptide. The digest has been officially approved as FOSHU in Japan (Fujita and Yoshikawa, 1999).

Table 10. ACE inhibitory activity (%) and antihypertensive effect in SHR from whey protein after digestion with one of seven proteases (Abubakar *et al.*, 1998).

Sample[1]	ACE inhibitory activity (%)	Decreased SBP[2] (mm Hg)
whey protein (control)	0.0	-38 ± 2
pepsin	83.7	-47 ± 3
trypsin	56.7	-51 ± 4*
α-chymotrypsin	76.0	-40 ± 4
proteinase K	95.7	-55 ± 3**
actinase E	55.7	-55 ± 4**
thermolysin	98.6	-42 ± 4
papain	86.5	-47 ± 4

[1]Dose was 8 mg/kg BW.
[2]The decrease in systolic blood pressure (SBP) 6 h after gastric intubation is shown as mean ± SE (n = 3).
*Different from control ($p < 0.05$).
**Different from control ($p < 0.01$).

3.3.4. Gastrointestinal proteases

ACE inhibitory peptides have also been produced by gastrointestinal proteases in sequential digestion. The ACE inhibitory activity of buckwheat protein is not enhanced by pepsin treatment, but a digest produced by pepsin, followed by trypsin and α-chymotrypsin, results in a significant increase in the ACE inhibitory activity and is able to lower the systolic blood pressure of SHR (Li *et al.*, 2002). The digestion of α-lactalbumin and β-lactoglobulin by pepsin, trypsin, α-chymotrypsin, elastase or carboxypeptidase A and B alone and in combination, reveals that trypsin is necessary to release high ACE inhibitory activity from whey protein (Pihlanto-Leppälä *et al.*, 2000). The gastrointestinal protease elastase, on the other hand, is associated with a low production of ACE inhibitory peptides from α-lactalbumin and β-lactoglobulin (Mullally *et al.*, 1997a; Pihlanto-Leppälä *et al.*, 2000). Pepsin digestion raises the ACE inhibitory activity of royal jelly protein considerably and subsequent hydrolysis by trypsin and α-chymotrypsin further reduces the IC_{50} to 0.099 mg protein/ml. Single oral administration of this gastrointestinal hydrolysate at 1 g/kg BW in SHR results in a significant lowering of the systolic blood pressure by 23 mmHg (Matsui *et al.*, 2002c). Three novel ACE inhibitory peptides have also been isolated from a tryptic digest of human α_{s1}-casein, which was expressed in *E. coli* and purified (Kim *et al.*, 1999).

3.3.5. Membrane reactors

A more industrial approach for the enzymatic production of ACE inhibitory and other bioactive peptides is the digestion in membrane reactors. In enzymatic membrane reactors, hydrolysis of the food proteins is combined with the purification of the bioactive peptides from the reaction mixture by filtration or precipitation. Moreover, a complete retention of the enzyme within the system makes the continuous operation of the membrane reactor feasible. Ultrafiltration membranes (1-100 nm or MWCO 500-100 000 Da) are most adequate for the retention of the majority of enzymes (Prazeres and Cabral, 1994). The major advantages of membrane reactors are the higher productivity due to the continuous process and the improved conversion in product-inhibited reactions, and the control of the molecular weight of the hydrolysates. A major disadvantage is the reduction in membrane filtration capacity during operation due to concentration polarisation and fouling and enzyme inactivation (Perea and Ugalde, 1996).

Tryptic hydrolysis of CMP is carried out in a continuous membrane reactor for the preparation of casoplatelines. An ultrafiltration membrane with MWCO 3000 Da continuously extracts four small bioactive peptides from the reactor and new substrate is fed in at the same rate. Compared to the batch reactor, the productivity of the continuous system is three times higher after 3.5 h of hydrolysis. However, CMP hydrolysis is about 50%, necessitating the optimisation of substrate concentration and flux and the insertion of a bleeding step for the membrane (Bouhallab *et al.*, 1992). In another study of the same group, the immunomodulatory peptide β-casein (193-209) is selectively isolated from the chymosin digest of β-casein. When the reactor is equipped with a cellulosic type membrane, the most satisfactory transmission of the peptide is obtained (Bouhallab *et al.*, 1993). It appears that continuous isolation of specific peptide(s) from reaction mixtures requires the use of a highly specific enzyme, the rapid release of the desired peptide, a large molecular weight difference between bioactive peptides and others to promote selective separation and a high transmission rate of the bioactive molecules through the ultrafiltration membrane.

In batch, oligopeptides of 1000 Da or less are obtained by hydrolysis of chicken egg yolks with the crude enzyme Newlase F and by subsequent dialysis with a semi-permeable membrane filter. In a long-term study, these extracted oligopeptides suppress the development of hypertension after oral administration in SHR (Yoshii *et al.*, 2001).

Using a three-step ultrafiltration membrane reactor, ACE inhibitory peptides are produced from skin gelatine from cow and Alaska Pollack by sequential protease treatment in the order of alkalase, pronase E and collagenase (Figure 11).

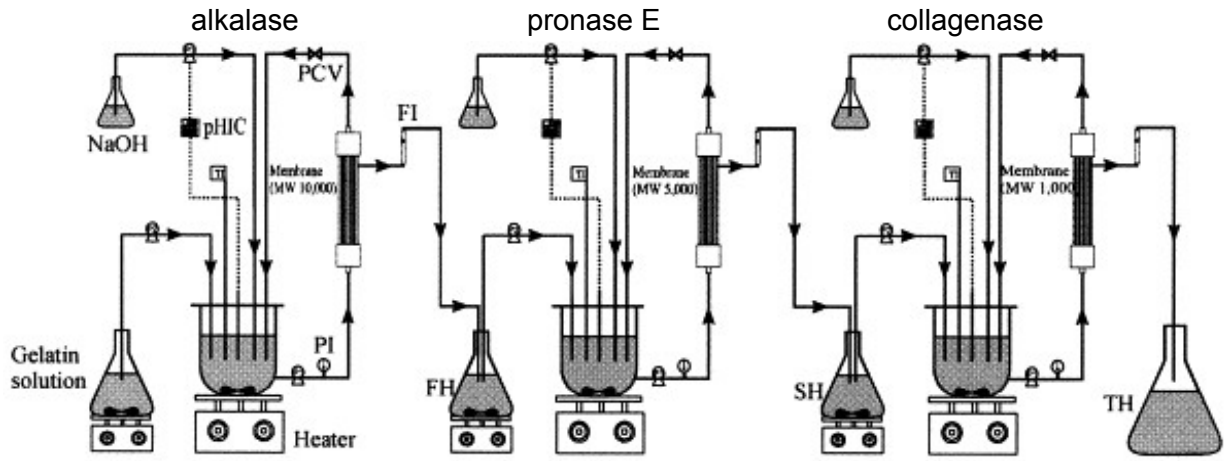


Figure 11. Schematic diagram of the three-step recycling membrane reactor for the production of enzymatic hydrolysates of gelatine. TI: temperature indicator; PI: pressure indicator; FI: flow indicator; P1: recycling pump; P2: feed pump; P3: NaOH pump; PCV: pressure control valve; pHIC: pH indicator controller; FH: first hydrolysate; SH: second hydrolysate; TH: third hydrolysate (Byun and Kim, 2001).

The ultrafiltration membranes from the first to the third reactor have a decreasing MWCO in the order of 10 000 Da, 5000 Da and 1000 Da. The combination of different enzymes results in a high degree of hydrolysis. For the fragmented hydrolysate, the ACE inhibitory activity markedly increases with decrease of MWCO of the membranes. For Alaska Pollack skin gelatine, the IC_{50} value of the third hydrolysate (0.63 mg/ml) is increased twofold compared with the value of the first hydrolysate (1.40 mg/ml). The third hydrolysate contains the ACE inhibitory peptides Gly-Pro-Met and Gly-Pro-Leu (Byun and Kim, 2001). For bovine skin gelatine, similar IC_{50} values are obtained for the hydrolysates and two potent ACE inhibitory peptides, Gly-Pro-Val and also Gly-Pro-Leu are purified from the third hydrolysate (Kim *et al.*, 2001).

3.4. Chemical synthesis

Based on known peptide sequences in food proteins, ACE inhibitory peptides may be synthesised chemically, which is expensive and not relevant for industrial production processes. However, recent advances in the design and construction of synthetic peptides enable the investigation of modifying functional activities (Mayo, 2000), which may have important implications for biotechnology.

3.5. Recent techniques

Production and isolation of bioactive peptides may be combined in one step. Chromatographic media together with *in situ* enzymatic hydrolysis and followed by selective elution enable the release of specific peptide sequences from a crude protein (Figure 12). Therefore it is essential to know the structural properties of these peptides. Anionic and cationic peptides with different biological activities can be isolated in this way. For example, lactoferrin is selectively bound when cheese whey is filtered through a cation-exchange membrane. Subsequently, it is directly hydrolysed *in situ* with pepsin. Inactive fragments are washed off the membrane with ammonia and a fraction enriched in the antimicrobial peptide lactoferricin is obtained by further elution with NaCl. This method has also proven to be effective in the isolation of phosphopeptides from milk proteins. Major advantages of this method are that the precursor protein does not need to be purified separately and that the other proteins in the crude mixture remain intact for other applications (Recio and Visser, 1999; Recio *et al.*, 2000).

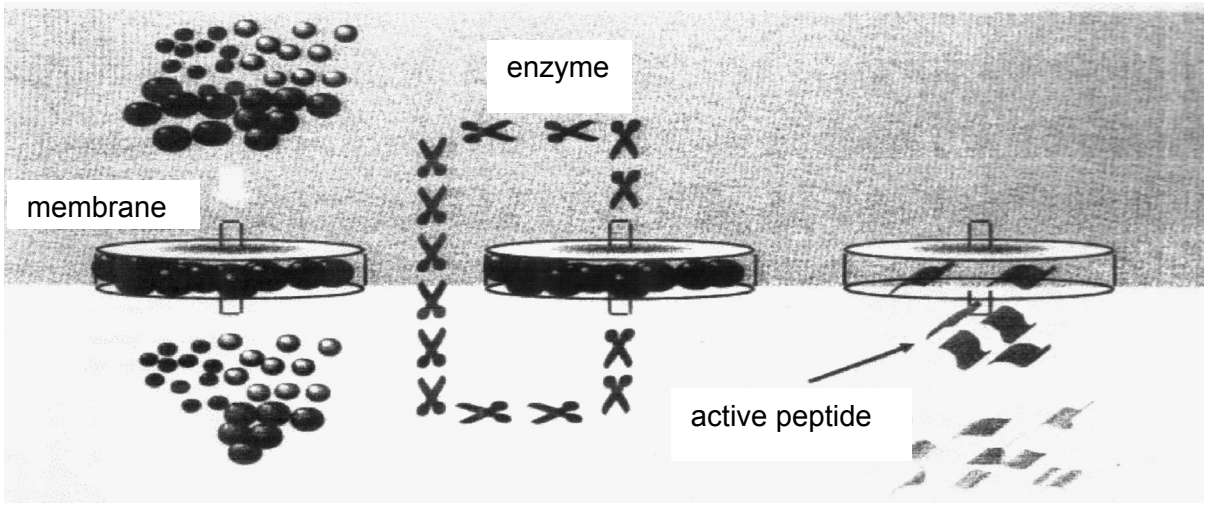


Figure 12. Method for the combined *in situ* hydrolysis and isolation of bioactive peptides (Floris, 2000).

Another approach is to chemically synthesise the oligonucleotides encoding ACE inhibitory peptides and express them in microorganisms. For example, oligonucleotides encoding the ACE inhibitory peptides Ile-Tyr and Val-Lys-Tyr are chemically synthesized and designed to be multimerised due to isoschizomer sites. The cloned gene, named *ap3*, is multimerised up to 6 times in a plasmid and expressed as a fusion protein, which is easily

purified. The digest of AP3 by chymotrypsin exhibits an IC_{50} value of 18.53 µM. Therefore, potent ACE inhibitory peptides may be released upon oral ingestion of AP3 (Oh *et al.*, 2002).

3.6. Isolation and characterisation of ACE inhibitory peptides

Various procedures are available to further purify and characterise bioactive peptides from hydrolysates. Firstly, usually a fractionation based on selective precipitation, ultrafiltration, chromatography or solid phase extraction is carried out to obtain a potent bioactive peptide mixture. Then, this is further purified to pure peptides by reversed phase - high performance liquid chromatography (RP-HPLC) (Herraiz, 1997). Different methods of mass spectrometry facilitate the detection and identification of peptides (Léonil *et al.*, 2000).

Precipitation of large peptides and proteins is performed with solutions containing organic solvents (methanol, ethanol or acetone) or acids (trichloroacetic acid), also by high concentration of salts (ammonium sulphate) or by adjusting the pH to the iso-electric point. Ultrafiltration is a reliable and simple method for fractionating peptides in function of their molecular size. Size exclusion chromatography or gel filtration also separates peptides according to molecular size. Ion exchange chromatography selects peptides based on their ionic character. Peptides containing very specific functional or active groups can be isolated by affinity chromatography. Solid phase extraction uses cartridges and columns with several bonded phases that isolate peptides by non-polar, polar and ion exchange mechanisms (Herraiz, 1997).

From a tryptic casein hydrolysate, hydrophobic peptides can be precipitated by a combination of pH and salt. As these peptides are often bitter, this procedure may be applicable to improve the taste of protein hydrolysates. On the other hand, it can be used to isolate bioactive peptides (Léonil *et al.*, 1994).

After tryptic digests of β-lactoglobulin and whey protein concentrate are subjected to ultrafiltration, the highest ACE inhibitory activity is present in the 3 kDa permeates as opposed to 1 and 10 kDa permeates and the unfractionated hydrolysates (Mullally *et al.*, 1997a). In the characterisation of whey protein hydrolysates digested by gastrointestinal proteases, the ACE inhibitory activity in the 1 kDa permeate is in many cases higher than in the retentate (Pihlanto-Leppälä *et al.*, 2000). Hence, ultrafiltration may be exploited to enrich for ACE inhibitory peptides.

When characterising oligopeptides from yoghurt, which contain bioactive peptide sequences, cation exchange separates uncharged and anionic compounds such as sugars or lactic acid, while subsequent ultrafiltration permits to isolate oligopeptides with molecular weight < 2 kDa. Final separation of oligopeptides is carried out by RP-HPLC on C_{18} silica

columns using gradient elution with an aqueous trifluoroacetic acid/acetonitrile mixture and UV detection (Schieber and Brueckner, 2000).

Two potent ACE inhibitory peptides were purified from skin gelatine hydrolysate by sequential chromatographic methods including size exclusion chromatography, ion exchange chromatography and RP-HPLC. During the successive purification steps the IC_{50} value of the active fraction decreased by a factor 400 (Byun and Kim, 2001; Kim *et al.*, 2001).

4. BIOAVAILABILITY OF ACE INHIBITORY PEPTIDES

4.1. Introduction

To exert physiological effects *in vivo* after oral ingestion, it is of crucial importance that ACE inhibitory peptides remain active during gastrointestinal digestion and absorption and reach the cardiovascular system. ACE inhibitory peptides may be released and degraded in the human body. Figure 13 shows the potential barriers in the human body where bioactive peptides can be (in)activated. This partially explains the discrepancy that is often observed between the ACE inhibitory activity of a food protein hydrolysate and its antihypertensive effect after oral administration (Table 6, 7 and 8). It is also suggested that ACE inhibitory peptides may exert an additional antihypertensive effect by inhibition of chymase and the endothelin converting enzyme (Okitsu *et al.*, 1995; Yamamoto *et al.*, 1999).

4.2. Gastrointestinal digestion

Digestion of proteins starts in the stomach by the action of pepsins at acidic pH. In humans the endopeptidases pepsin A and C are present, which exhibit similar substrate specificity (Table 9). In the luminal phase of the small intestine, the polypeptides are further cleaved by the pancreatic proteases trypsin, α-chymotrypsin, elastase, carboxypeptidase A and B at more alkaline pH (Table 9). This results in a mixture of oligopeptides and free amino acids, of which oligopeptides constitute a major portion. The free amino acids are absorbed as such into the enterocytes across the brush border membrane via distinct amino acid transport systems. The oligopeptides undergo further hydrolysis by the action of a battery of brush border peptidases resulting in a mixture largely consisting of free amino acids, di- and tripeptides. The intestinal brush border membrane is particularly rich in aminopeptidase activity, which provides functional complementation to the carboxypeptidases present in the pancreatic juice. Aminopeptidase N and A are the major representatives and cleave N-terminal neutral and anionic amino acids, respectively.

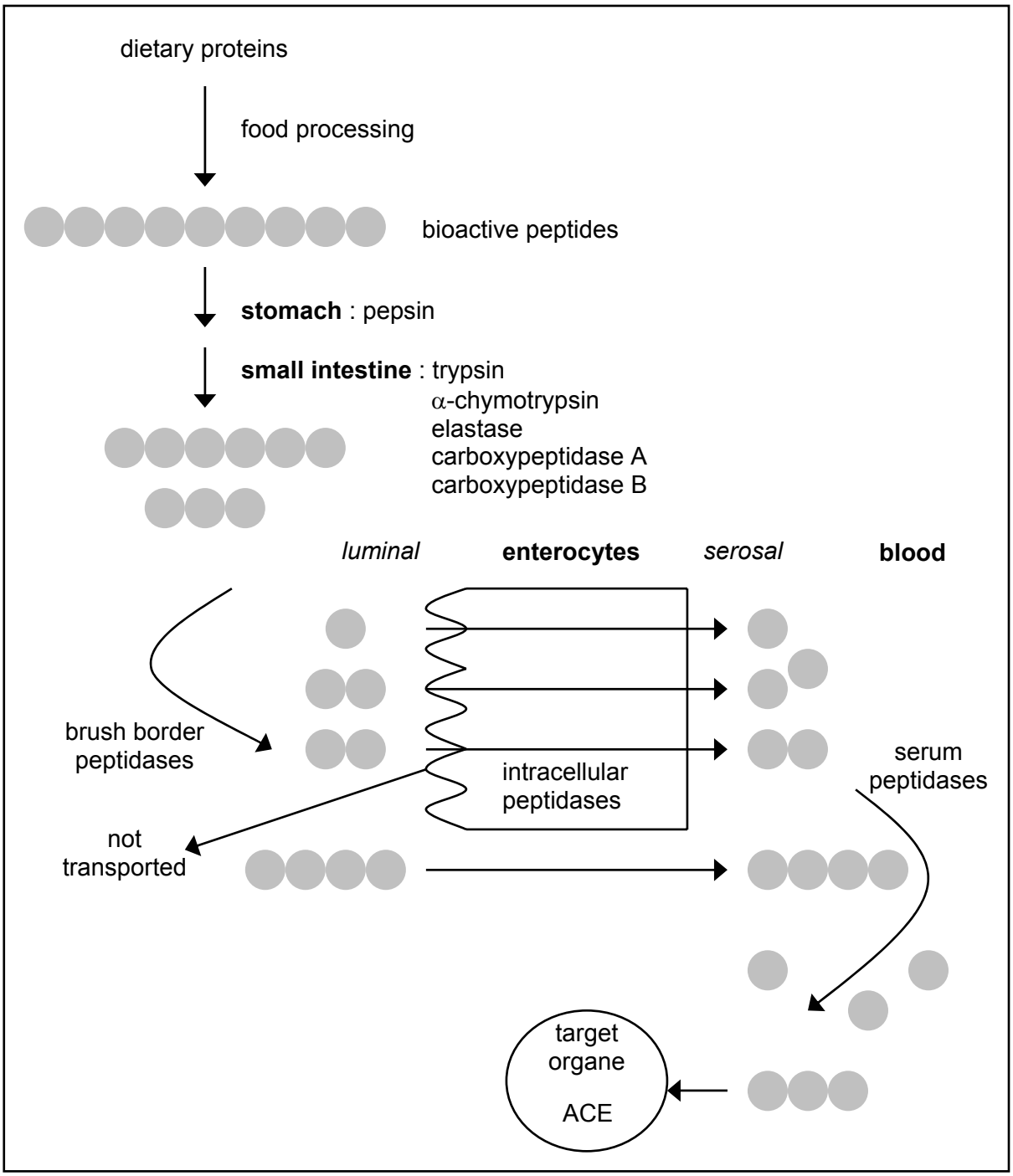


Figure 13. The potential activation and inactivation of ACE inhibitory peptides in the human body during gastrointestinal digestion, absorption and in the blood.

In addition to these enzymes, the intestinal brush border contains endopeptidase and dipeptidase activity. ACE or dipeptidyl carboxypeptidase cleaves dipeptides from the C-terminus of oligopeptides with Pro, Phe or Leu in the ultimate position. Dipeptidyl aminopeptidase IV releases dipeptides from the N-terminus of oligopeptides with Pro or Ala

in the penultimate position. Hence, the dipeptides released by these enzymes are generally of the X-Pro type (Ganapathy and Leibach, 1999; Ganong, 1997a).

Proline and hydroxyproline containing peptides are generally resistant to degradation by digestive enzymes. Furthermore, tripeptides containing C-terminal Pro-Pro are reported to be resistant to proline specific peptidases (Fitzgerald and Meisel, 2000; Vanhoof *et al.*, 1995). This stresses the fact that several bioactive peptides that have been shown to exert an *in vivo* effect, are isolated from casein and gelatine, as these proteins contain a high amount of proline.

Bioactive peptides have already been characterised from *in vivo* digests. When diets containing bovine casein are given to minipigs, the opiate peptide β–casomorphin-11 and the CPP α_{s1}-casein f(66-74) are isolated from small intestinal contents (Meisel and Frister, 1989). In another study, β-casomorphin-7 immunoreactive material is detected in small intestinal contents of adult humans after bovine milk ingestion (Svedberg *et al.*, 1985). In adult humans, after milk or yoghurt ingestion, the appearance of bioactive peptides in the stomach, small intestine and blood has been investigated. Many peptides derived from α_{s1}-, β- or κ-caseins have been detected in the stomach, smaller peptides derived from casein and lactoferrin have been recovered from the small intestine. Two long peptides, CMP, κ-casein f(106-117) and the N-terminal peptide f(1-23) of α_{s1}-casein, are absorbed and detected in plasma (Chabance *et al.*, 1998). Hence, casoplatelines (CMP fragments) are released during gastrointestinal digestion and absorbed intact into the blood, which supports the concept that they exert an antithrombotic effect *in vivo*. The peptide Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys-Glu, α_{s1}-casein f(24-35), and f(25-32) are released in the human stomach. The latter fragment and f(28-35) are also detected in the duodenum. As these contain sequences of ACE inhibitory peptides (Table 6) (Karaki *et al.*, 1990; Maruyama *et al.*, 1987b), they might exert a physiological effect after absorption in the blood. The peptide α_{s1}-casein f(1-23) is the antibacterial peptide isradicin with immunomodulatory capacities. This includes the fragment α_{s1}-casein f(1-9), Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln, which has shown to have a small antihypertensive effect in SHR (Table 6) (Saito *et al.*, 2000).

To investigate if ACE inhibitory peptides resist gastrointestinal digestion, they are usually subjected *in vitro* to hydrolysis by pepsin, trypsin, α-chymotrypsin or pancreatin. For example, ACE inhibitory wheat germ hydrolysate and the peptide Ile-Val-Tyr isolated from it, are hydrolysed by pepsin, trypsin and α-chymotrypsin alone and in combination. The ACE inhibitory activity of the wheat germ hydrolysate increases with 27% after a combined digestion, indicating that any active peptides must be newly produced by action of these proteases, in particular trypsin. The IC_{50} value of Ile-Val-Tyr does not change during

digestion, signifying that this peptide would resist *in vivo* gastrointestinal digestion (Matsui *et al.*, 1999). Another example of activation of ACE inhibitory peptides during gastrointestinal digestion is the following. The antihypertensive peptide Lys-Val-Leu-Pro-Val-Pro-Gln shows only low ACE inhibitory activity *in vitro*. However, the potent ACE inhibitor Lys-Val-Leu-Pro-Val-Pro is formed after pancreatic digestion due to the action of carboxypeptidase A. In the same study, an α_{s1}-casein derived peptide, Tyr-Lys-Val-Pro-Gln-Leu, with strong ACE inhibitory activity, fails to exert an antihypertensive effect due to pancreatin degradation (Maeno *et al.*, 1996).

After the small intestine, the non-digested and/or non-absorbed food peptides enter the large intestine or colon, where they can be metabolised by the intestinal microbiota. Here, hundreds of bacterial species interact with one another and the host, forming an integral and biologically important component of the body. The normal intestinal microbiota may possess a range of beneficial features from an impact on the morphology and physiology of the intestinal tract (Falk *et al.*, 1998), over the suppression of colonization by pathogens and opportunistic microorganisms (Anonymous, 1999), the metabolisation of a variety of compounds and the production of certain vitamins and fatty acids, to the stimulation of the immune system (Iijima *et al.*, 2001). However, the normal microbiota is also involved in the metabolism of some potentially harmful substances leading to intestinal putrefaction and an increased risk of colon cancer (Knasmuller *et al.*, 2001). To study the gastrointestinal microbial ecosystem and its impact on the health and well-being of the host, *in vitro* systems have been designed to reproduce the main nutritional and environmental conditions that affect the composition and functionality of the complex ecosystem (Rumney and Rowland, 1992). One example is the SHIME, Simulator of the Human Intestinal Microbial Ecosystem (Molly *et al.*, 1994). This semi-continuous *in vitro* system consists of five reactor vessels, simulating the different parts of the gastrointestinal tract, respectively stomach, small intestine, caecum + colon ascendens, colon transversum and colon descendens. At the start-up the system was inoculated with fecal material. By addition of an adapted diet, pancreatic juice and bile, and by controlling pH, temperature and retention time in the different vessels, the microbiota of the human individual can be mimicked. The first two vessels work semi-continuously with a fill-and-draw system, while in the last three vessels, simulating the large intestine, the suspension is continuously transferred. By selective adaptation of the system, a simulator for the infant (baby-SHIME) (De Boever *et al.*, 2001) and pig (SPIME) microbial community have also been created. Recently the SHIME apparatus was extended by a computer control system. The SHIME has been demonstrated to be very useful in examining the intestinal microbial ecology and in studying the effect of functional foods on the gastrointestinal microbial community (De Boever *et al.*, 2000).

4.3. Intestinal absorption

Peptides consisting of two or three amino acids are absorbed intact across the brush border membrane by a specific peptide transport system. The peptide transporter PepT1 uses a transmembrane electrochemical proton gradient as the driving force and has broad substrate specificity (Yang *et al.*, 1999). Small peptides are absorbed more rapidly than free amino acids and their transport is favoured (Webb, 1990). Once inside the enterocyte, these peptides are usually hydrolysed to free amino acids in the cytoplasm by various intracellular peptidases. Among these, aminotripeptidase, which releases the amino acid from the N-terminus of tripeptides, and several dipeptidases with different substrate specificity towards specific amino acids, are present. Here, iminodipeptidase or prolidase is of special interest because of its restricted specificity toward dipeptides of the X-Pro or X-hydroxyproline type. Via specific amino acid transport systems, the amino acids cross the basolateral membrane and enter the portal circulation (Ganapathy and Leibach, 1999).

Transport of intact peptides and proteins from the intestinal lumen into the blood circulation is a unique phenomenon, which differs from the regular process of food digestion and absorption. The concept that significant amounts of small peptides can escape total digestion to amino acids and enter the circulation in intact form is rather new, but is gaining acceptance (Gardner, 1988; Grimble, 2000). Apart from the peptide transporter route, peptides can be absorbed intact across the intestinal mucosa via other mechanisms (Figure 14) (Gardner, 1998). There is evidence in support of both paracellular and transcellular routes for passage of intact peptides, but there is still debate as to the relative importance of these. Paracellularly, large water-soluble peptides pass via the tight junctions between cells. Highly lipid-soluble peptides appear to be able to diffuse via the transcellular route. Peptides may also enter the enterocytes via endocytosis which entails membrane binding and vesiculisation of the material (Ziv and Bendayan, 2000). The intestinal basolateral membrane also possesses a peptide transporter, which facilitates the exit of hydrolysis-resistant small peptides from the enterocyte into the portal circulation (Gardner, 1984).

Biologically active peptides generated in the diet can be absorbed intact through the intestine and produce biologic effects at the tissue level. However, the potency of the administered peptides decreases as the chain length increases (Roberts *et al.*, 1999). As in infants the gastrointestinal barrier is not yet completely mature, intact peptides and proteins are much better absorbed in infants than in adults (Walker, 1985). Together with the limited gastrointestinal proteolysis in infants, this explains why milk contains several bioactive proteins and peptides that are presumed to have a physiological role in the young.

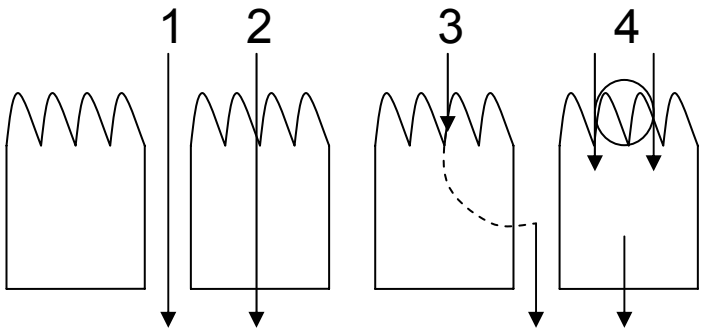


Figure 14. Different mechanisms for intestinal transport of peptides: 1) paracellular, 2) passive diffusion, 3) endocytosis, 4) carrier-mediated transport.

In this respect, β-casomorphin immunoreactive material is detected in the plasma of newborn calves after milk intake (Umbach *et al.*, 1985). Higher plasma levels of CMP are found in 5-day old infants compared to human adults after ingestion of milk (Chabance *et al.*, 1995; Chabance *et al.*, 1998). In the latter study, it is demonstrated that a peptide containing 24 amino acids is absorbed intact into the blood stream of human adults after milk ingestion (see above) (Chabance *et al.*, 1998). Intestinal transport of β-casomorphins and synthetic analogues is investigated in isolated rabbit ileum mounted in Ussing chambers. While natural β-casomorphins are degraded by the intestinal mucosa and no intact transpepithelial passage is detected for these peptides, the synthetic analogue Tyr-D-Ala-Phe-D-Ala-Tyr crosses the epithelium intact (Tome *et al.*, 1987).

When the intestinal transport mechanism of the antihypertensive peptide Val-Pro-Pro is studied in a Caco-2 cell monolayer, a low but significant amount of intact Val-Pro-Pro is transported through the cell monolayer. The transport is not mediated by the peptide transporter PepT1 and it is suggested that paracellular transport is the main mechanism of absorption (Satake *et al.*, 2002). In another paper, the transepithelial transport of the oligopeptides bradykinin, β-casomorphin Tyr-Pro-Phe-Pro-Gly, ovokinin Phe-Arg-Ala-Asp-His-Pro-Phe-Leu, Pro-Phe-Gly-Lys and Gly-Gly-Tyr-Arg is investigated in the Caco-2 model. The susceptibility to the brush border peptidases is one of the factors that decide the transport rate. Bradykinin and Gly-Gly-Tyr-Arg are hardly degraded, while β-casomorphin and ovokinin are hydrolysed substantially. The transport rate is greatest for Gly-Gly-Tyr-Arg and smallest for β-casomorphin. Furthermore, the transport mechanism is studied for bradykinin and Gly-Gly-Tyr-Arg. While adsorptive transcytosis through hydrophobic interaction with the cell membrane is suggested to be involved in the transport of bradykinin, Gly-Gly-Tyr-Arg is believed to be transported mainly via the paracellular pathway. It seems

that molecular size and other structural properties like hydrophobicity determine the major transport way for peptides (Shimizu *et al.*, 1997).

The antihypertensive peptide Val-Tyr, derived originally from sardine muscle, is dose-dependently detected in human plasma and reaches a maximum 2 h after a single oral administration in normotensive human subjects. At the highest dose, more than a tenfold higher increment in Val-Tyr plasma concentration is measured compared to the baseline concentration. The low absorption ratios are explained by the fact that most of the administered dipeptide is hydrolysed by membrane-bound peptidases. Therefore, it is suggested that saturation of the peptide transporter enables the entry of excess Val-Tyr into peripheral blood (Matsui *et al.*, 2002a).

4.4. Stability in the blood

Blood contains substantial activities of peptidase enzymes. The half-life of certain peptides in plasma is very short, with an order of magnitude of 1 minute (Gardner, 1998). Angiotensin II degradation occurs even within seconds (Moskowitz, 2003).

First of all, oligopeptides with an ACE inhibitory activity *in vitro*, need to resist ACE to exert an antihypertensive effect *in vivo*. In this respect, the ACE inhibitory peptides can be classified in three groups: the inhibitor type, of which the IC_{50} values are not affected by preincubation with ACE; the substrate type, peptides that are hydrolysed by ACE to give peptides with a weaker activity and the prodrug-type inhibitor, which are converted to true inhibitors by ACE or other gastrointestinal proteases. Only peptides belonging to the inhibitor or pro-drug type exert antihypertensive activities after oral administration in SHR (Fujita *et al.*, 2000).

After intravenous administration of the ACE inhibitory peptide Ile-Val-Tyr, isolated from wheat germ hydrolysate, to SHR, the tripeptide is observed to be metabolised by the action of aminopeptidase in plasma to form a subsequent ACE inhibitor, Val-Tyr. The latter peptide exerts an acute depressor effect in SHR and the blood pressure returns about 5 min after injection to the normal state. On the other hand, after injection of Ile-Val-Tyr, the reduction in blood pressure is much stronger and is held for 15 min. Therefore, it is suggested that the intake of Ile-Val-Tyr as a physiologically functional food would serve in lowering the blood pressure by the combined action of itself and its metabolite after absorption (Matsui *et al.*, 2000). Val-Tyr has been administered orally to mild hypertensive subjects. Although the peptide is present in plasma and the absorption is comparable to the one observed in normotensive subjects (see above), no marked decrease in blood pressure is seen, suggesting that the peptide exerts no acute hypotensive effect (Matsui *et al.*, 2002b).

In conclusion, the bioavailability of ACE inhibitory peptides is predominantly determined by the resistance to peptidase degradation and intestinal absorption. *In vivo* experiments and clinical trials are necessary to demonstrate their physiological effects.

5. GOALS AND OUTLINE OF THE STUDY

Hypertension or high blood pressure is a major risk factor for cardiovascular diseases, which are the leading causes of morbidity and mortality in western society. Lifestyle modifications and diet therapy, such as the DASH diet, have been shown to be effective in both the prevention and the treatment of hypertension. In this respect, bioactive peptides with antihypertensive properties like ACE inhibitory peptides may play a functional role as part of a functional food or as nutraceutical. As they are less active than their drug counterparts, they have not yet demonstrated any side effects. Moreover, they are less expensive.

ACE inhibitory peptides have mainly been isolated from milk proteins and have not yet been studied from pea protein. Whey has high biological value and originates as by-product from milk during the cheese making process. Pea is a long established and significant crop in Europe, but is nowadays mainly used in animal nutrition, although it has some advantages for human nutrition as well. Pea protein has a well balanced profile of amino acids, and especially a high content in lysine. Moreover, in accordance with today's consumer demands, it is environment-friendly produced: not only can it present a non-GMO alternative to soy protein, but it is also a low input crop due to symbiotic nitrogen fixation.

This thesis aimed to investigate the formation of ACE inhibitory activity from pea and whey protein and to compare whey protein, from which certain bioactive peptides already have been characterised, to the unknown pea protein. Emphasis was placed on the *in vitro* study of the activity of ACE inhibitory peptides after oral administration in the human body, from the gastrointestinal tract to their site of action, the cardiovascular system. This was done by means of an *in vitro* gastrointestinal digestion, by simulation of the brush border phase and intracellular enterocyte enzyme activity and by *in vitro* intestinal transport studies. Literature review indicated that the maintenance of biological activity during the oral delivery route is often neglected.

More specifically, the goals of this work were:

- To optimise and validate an ACE inhibition assay, which is used to measure the biological activity of ACE inhibitory peptides *in vitro* and facilitates the screening of such peptides from food proteins (Chapter 2).
- To study the activity of the potent lactokinin Ala-Leu-Pro-Met-His-Ile-Arg in the oral delivery route *in vitro*: stability towards gastrointestinal proteases and peptidases in the brush border and enterocytes, intestinal transport and antihypertensive effect (Chapter 3).
- To release ACE inhibitory activity from pea and whey protein by fermentation with GRAS microorganisms and to study the influence of an *in vitro* gastrointestinal digestion on this activity (Chapter 4).
- To characterise the conditions during *in vitro* gastrointestinal digestion of pea and whey protein leading to maximal ACE inhibitory activity release. This was investigated in batch by comparing three different *in vitro* digestions and in a semi-continuous reactor model by response surface methodology (Chapter 5).
- To study the evolution of ACE inhibitory activity and protein degradation during an *in vitro* gastrointestinal physiological digestion, subsequently supplemented with rat intestinal acetone powder. Via an ACE inhibitory peptide database, protein databases and protein cleavage software, the ACE inhibitory activity and protein degradation during gastrointestinal digestion was also investigated *in silico* (Chapter 6).
- To enrich the ACE inhibitory activity in pea and whey digests by ultrafiltration and RP-HPLC fractionation, investigate the *in vitro* intestinal transport of these digests and permeates and examine the antihypertensive activity of pea and whey permeate in SHR (Chapter 7).

The major findings of this work are summarised and discussed in Chapter 8. In addition, some perspectives for ACE inhibitory peptides are mentioned.

CHAPTER 2

AN ANGIOTENSIN I CONVERTING ENZYME INHIBITION ASSAY FOR THE SCREENING OF BIOACTIVE PEPTIDES

Redrafted after VERMEIRSSEN, V., VAN CAMP, J. & VERSTRAETE, W. (2002b). Optimisation and validation of an Angiotensin Converting Enzyme (ACE) inhibition assay for the screening of bioactive peptides. *Journal of Biochemical and Biophysical Methods*, 51:75-87.

Chapter 2

An angiotensin I converting enzyme inhibition assay for the screening of bioactive peptides

ABSTRACT

Angiotensin I converting enzyme (ACE) plays a major role in the regulation of blood pressure. A diagnostic assay to measure ACE activity was transformed into an enzyme inhibition assay and optimised, which led to a more sensitive and less expensive assay. By this spectrophotometric method, ACE inhibition is measured using the substrate furanacryloyl-Phe-Gly-Gly and rabbit lung acetone extract as ACE source. The optimised as well as the original ACE inhibition assays were used to verify the ACE inhibitory activity of captopril. The ACE inhibition assay was further validated by enalapril, its active derivative enalaprilat and the ACE-inhibitory peptide Ala-Leu-Pro-Met-His-Ile-Arg, corresponding to a tryptic fragment of bovine β-lactoglobulin. Sigmoid curves could be fit adequately to the data points representing ACE inhibition in function of inhibitor concentration. IC_{50} values for these compounds corresponded well with literature data. Futhermore, pea and whey protein hydrolysates obtained by digestion with trypsin showed ACE inhibitory activity in the ACE inhibition assay. Hence, this optimised assay is suited for the screening of ACE inhibitory peptides derived from food proteins with a possible antihypertensive effect *in vivo*. Later on, a more delicate and standardised assay was obtained by applying pure ACE from porcine kidney. This assay was also validated by captopril and Ala-Leu-Pro-Met-His-Ile-Arg. For most experiments done in this work, ACE inhibitory activity was measured by the latter assay.

Keywords: Angiotensin I Converting Enzyme (ACE), ACE inhibition assay, bioactive peptides, IC_{50}, whey protein, pea protein

INTRODUCTION

Angiotensin I converting enzyme (ACE; EC 3.4.15.1) is a non-specific dipeptidyl carboxypeptidase associated with the blood pressure regulating renin-angiotensin system. This enzyme increases blood pressure by converting the decapeptide angiotensin I into the potent vasoconstricting octapeptide angiotensin II. Angiotensin II brings about several central effects all leading to a further increase in blood pressure. ACE is a multifunctional enzyme that also catalyses the degradation of bradykinin, a blood pressure lowering nonapeptide (Erdös and Skidgel, 1987; Johnston, 1992). Therefore, inhibition of the angiotensin I converting enzyme results in an overall antihypertensive effect.

Synthetic ACE inhibitors are one group of drugs in the treatment of hypertension. However, these synthetic drugs can have significant side effects due to their high activity and specificity (Messerli, 1999). On the other hand, peptides with ACE inhibitory activity have already been isolated from different food proteins (Ariyoshi, 1993; Dziuba *et al.*, 1999a; Yamamoto, 1997). These so-called 'biologically active peptides' could represent a healthier and natural alternative treatment of hypertension.

To measure ACE activity and inhibition *in vitro*, the method of Cushman and Cheung (1971) is often used. In this spectrophotometric assay, the extent of hippuric acid release from hippuryl-L-histidyl-L-leucine (HHL) is directly related to the ACE activity. Rabbit lung acetone extract is the enzyme source. Nakamura *et al.* (1995a) later modified this assay by using pure ACE from rabbit lung instead of rabbit lung acetone extract. However, a major drawback of this method is that the absorbance to determine the hippuric acid concentration can only be measured after extraction with ethyl acetate. Additionally, an incubation time of 30 min is necessary to yield a significant amount of product, which further prolongs the experiment. Sigma-Aldrich distributes a relatively fast ACE assay, based on the method of Holmquist *et al.* (1979) (Wong and Kinniburgh, 1987). Here, the ACE activity is determined by the blue shift of the absorption spectrum that occurs upon hydrolysis of a furanacryloyl tripeptide (FA-Phe-Gly-Gly, FAPGG) leading to the production of the corresponding amino acid (FA-Phe, FAP) and dipeptide (Gly-Gly, GG). The release of these peptides decreases the absorbance at the predescribed wavelength. The angiotensin I converting enzyme used in this assay originates from porcine kidney and is dissolved in human serum.

We have modified this assay to determine ACE inhibition by the addition of an inhibitory compound in the reaction mixture and optimised it by using rabbit lung acetone extract as ACE source and applying the appropriate enzyme-substrate ratio, which increased the sensitivity of the assay. The optimised and original ACE inhibition assays were both used to determine the well-known ACE inhibitory activity of captopril, an antihypertensive drug. The

optimised ACE inhibition assay was further validated with enalapril, an antihypertensive prodrug, its active derivative enalaprilat and Ala-Leu-Pro-Met-His-Ile-Arg, an ACE inhibitory peptide isolated from a tryptic digest of bovine β-lactoglobulin with an IC_{50} of 42.6 µM (Mullally *et al.*, 1997b). Digests of pea and whey protein obtained with trypsin (EC 3.4.21.4) were also assessed for ACE inhibitory activity by this protocol. Afterwards, this assay was slightly modified by using pure ACE from porcine kidney as enzyme source. The ACE inhibitory activity of captopril and Ala-Leu-Pro-Met-His-Ile-Arg were also measured by this method.

MATERIALS AND METHODS

Products

ACE reagent (305-10), ACE control-E (A 7040), rabbit lung acetone powder (L 0756), elastase (E 1250), trypsin (T 1426), Angiotensin I Converting Enzyme from rabbit lung (A 6778), captopril (C 4042), enalapril (E 6888) and Gly-Gly-Gly (G 1377) were purchased from Sigma-Aldrich (St.-Louis, MO, USA). The inhibitor solutions were prepared from 10^{-3} M stock solutions of captopril and enalapril in demineralised water and stored at -20°C. Angiotensin I Converting Enzyme from rabbit lung, reconstituted with demineralised water (0.1 U/ml), was kept at 5°C. The ACE inhibitory peptide Ala-Leu-Pro-Met-His-Ile-Arg was synthesised by solid phase peptide chemistry and FMOC protection at the Biotechnology Center of the University of Illinois, Urbana-Champaign, IL, USA.

The pea protein isolate Pisane® HD and the (rennet) whey protein isolate Lacprodan® DI-9213 were obtained from Cosucra SA (Fontenoy, Belgium) and Acatris Belgium NV (Londerzeel, Belgium), respectively. Non-specified products were analytical grade from VWR International (Zaventem, Belgium).

Sigma ACE assay transformed into an ACE inhibition assay: assay 1

The ACE reagent contains 0.005 mmol FAPGG with stabiliser and buffer to ensure that the reaction takes place at pH 8.1-8.3. It was reconstituted with 5 ml instead of 10 ml demineralised water in order to maintain the substrate concentration of $4.6 .10^{-4}$ M in the final reaction mixture after addition of a volume of inhibitory compound. ACE control-E was reconstituted with 1 ml demineralised water according to the distributor. This solution contains an assigned activity of porcine angiotensin I converting enzyme in a buffered human base with stabilizers and preservatives. Five hundred µl ACE reagent and 500 µl

demineralised water (blank) or inhibitor solution were mixed and pre-incubated for 2 min at 37°C. After addition of 100 µl enzyme (ACE control-E) to this suspension, the reaction mixture was incubated for 5 min at 37°C. Subsequently, the absorbance was measured against demineralised water in a heated cuvet holder (37°C) of an UV/VIS double beam spectrophotometer (Uvikon 922, Kontron Instruments, Watford Herts, UK) at 340 nm over a time interval of exactly 5 min. The pH of the reaction (8.1-8.3) was verified by means of indicator paper.

ACE inhibition assay with rabbit lung acetone extract: assay 2

The rabbit lung acetone extract was prepared by dissolving 1 g rabbit lung acetone powder in 10 ml 50 mM potassium phosphate buffer, pH 8.3 and ultracentrifuging the mixture for 40 min at 40 000 g (L 7-55 ultracentrifuge, Beckman, Fullerton, CA, USA). The clear wine red supernatant possessed high ACE activity and was kept at 5°C (Cushman and Cheung, 1971). Prior to the assay, the supernatant was diluted 10 times in 50 mM potassium phosphate buffer, pH 8.3.

ACE inhibitory activity was assayed with a variant of the above-described transformed Sigma assay. The reaction mixture contained 500 µl ACE reagent, 300 µl demineralised water (blank) or inhibitor solution and 300 µl ten times diluted rabbit lung acetone extract. The incubation and spectrophotometric determination of the mixture was identical to assay 1.

ACE inhibition assay with pure ACE from porcine kidney: assay 3

Instead of 300 µl ten times diluted rabbit lung acetone extract, 300 µl reconstituted ACE control-E was added as enzyme source. All other conditions were identical to assay 2. For most experiments done in this work, ACE inhibitory activity was measured by the latter assay. The concentration of the analysed inhibitory ferments and hydrolysates in the assay was 2.73 mg/ml when determining a percent ACE inhibitory activity (see next chapters).

Standard curve

The decrease in absorbance over 5 min corresponds to an ACE activity as determined by the standard curve. This curve was constructed using different dilutions of the ACE control-E as enzyme source. In assay 1 up to 100 µl pure ACE control-E was added, while in assay 2 and 3 this was 300 µl. Data were fitted by a linear regression model using the Marquardt-Levenberg algorithm (minimisation of the sum of the squared differences between the values of the observed and predicted values of the dependent variable) (Sigmaplot 8.0, SPSS Inc.,

Chicago, Illinois, USA). The activity of the angiotensin I converting enzyme is expressed in U/l, whereby 1 unit is defined as the amount of enzyme that catalyses the formation of 1 µmol FAP per min under the assay conditions of the Sigma-Aldrich ACE assay. Pure rabbit lung angiotensin I converting enzyme (0.1 U Cushman & Cheung/ml) was also once applied as enzyme source in assay 2 in order to compare the FAP units with the Cushman & Cheung units.

Inhibition

Inhibition was expressed as the concentration of inhibitory compound in the assay that inhibits 50% of ACE activity (IC_{50}), assuming that the activity of the blank is 100%. When ACE inhibitory activity exceeded 80%, dilution series were made to determine the IC_{50} value. Dose-activity curves were generated for doses of inhibitor (abscissa) versus ACE activity (ordinate). The data were fitted by a 4 parametric logistic model using the Marquardt-Levenberg algorithm (Sigmaplot 8.0, SPSS Inc., Chicago, Illinois, USA). The IC_{50} value was obtained from the parameters of the fitted function.

$$y = \min + \frac{\max - \min}{1 + 10^{[\log IC_{50} - x].hill\ slope}}$$

In this equation y represents the ACE inhibitory activity (%) and x the logarithm of the concentration of inhibitor (M or mg/ml). Parameter *min* equals the baseline of 100% inhibition, *max* the plateau of 100% activity. Parameter IC_{50} gives the transition center. The *hill slope* determines the slope of the curve at the transition center.

Chloride ion determination in the ACE reagent

Chloride ion concentration of thousand fold diluted ACE reagent was determined by the spectrophotometric LCK 311 assay on the ISIS 9000 apparatus (Dr. Lange, Düsseldorf, Germany).

Effect of elastase on FAPGG

Elastase solutions of 10 mg/1.5 ml and a tenfold dilution (1 mg/1.5 ml) in 50 mM potassium phosphate buffer, pH 8.3, were used as enzyme source in the ACE inhibition assay with rabbit lung acetone extract (assay 2). Instead of using 300 µl of the ten times diluted rabbit lung acetone extract, 300 µl of elastase solution was added in assay 2.

Chemical hydrolysis of enalapril to enalaprilat

Four ml 1 M NaOH was added to 10 ml 10^{-3} M enalapril solution in demineralised water and the reaction mixture was placed at room temperature overnight. Afterwards, the pH was adjusted to 8 with 1 M HCl and the solution was diluted to 20 ml with demineralised water. Assuming complete conversion, the final concentration enalaprilat in the solution amounted to $5 .10^{-4}$ M. Different dilutions of this solution were used in assay 2.

Preparation of ACE inhibitory samples

Pea protein and whey protein isolate (4% (w/v) in demineralised water) were digested with trypsin (E/S = 1/250 (w/w)) for 2 h at 37°C and pH 7. Samples were taken before trypsin addition and after digestion. In both cases, the pH was adjusted to pH 5 with 10 N and 1 N HCl or NaOH while heavily stirring the solution and samples were centrifuged for 15 min at 10 000 g. Before freezing the supernatant at -70°C, the protease was inactivated by cooling in liquid nitrogen for 25 s. After minimum 12 h the samples were lyophilised. Ten mg of the lyophilised powder was dissolved in 1 ml demineralised water and used as ACE inhibitory solution in ACE inhibition assay 2 (concentration of 2.73 mg/ml in the assay).

Statistical analysis

Values are reported as mean ± standard error (SE). Statistical analysis of the parameters was performed by the F-test ($p < 0.05$) to verify equality of variances and by the appropriate two-tailed Student t-test ($p < 0.05$; 0.01; 0.001) to determine whether two samples are likely to have come from the same two underlying populations that have the same mean.

RESULTS

Standard curve

In our ACE inhibition assays, ACE catalyses the degradation of the substrate, so that the ACE activity could be derived from the decrease in absorbance during the reaction. In the standardised assays, the absorbance was measured at 340 nm 5, 7.5 and 10 min after the addition of the enzyme. The decrease in absorbance for a given ACE concentration occurred linearly in time during the 5 min measurement. A standard curve was constructed using different dilutions of the ACE control-E (Figure 1). Regression analysis revealed a linear model with a slope of 0.0031 ± 0.0001, an intercept of 0.0110 ± 0.0013 and a correlation coefficient of 94% (n = 62). The detection limit is defined as the concentration that yields a decrease in absorbance that is still significantly different from the background signal. The detection limit of the ACE inhibition assays, predicted with 99.7% confidence, was 2.9 U/l. This corresponded to a decrease in absorbance of 0.02.

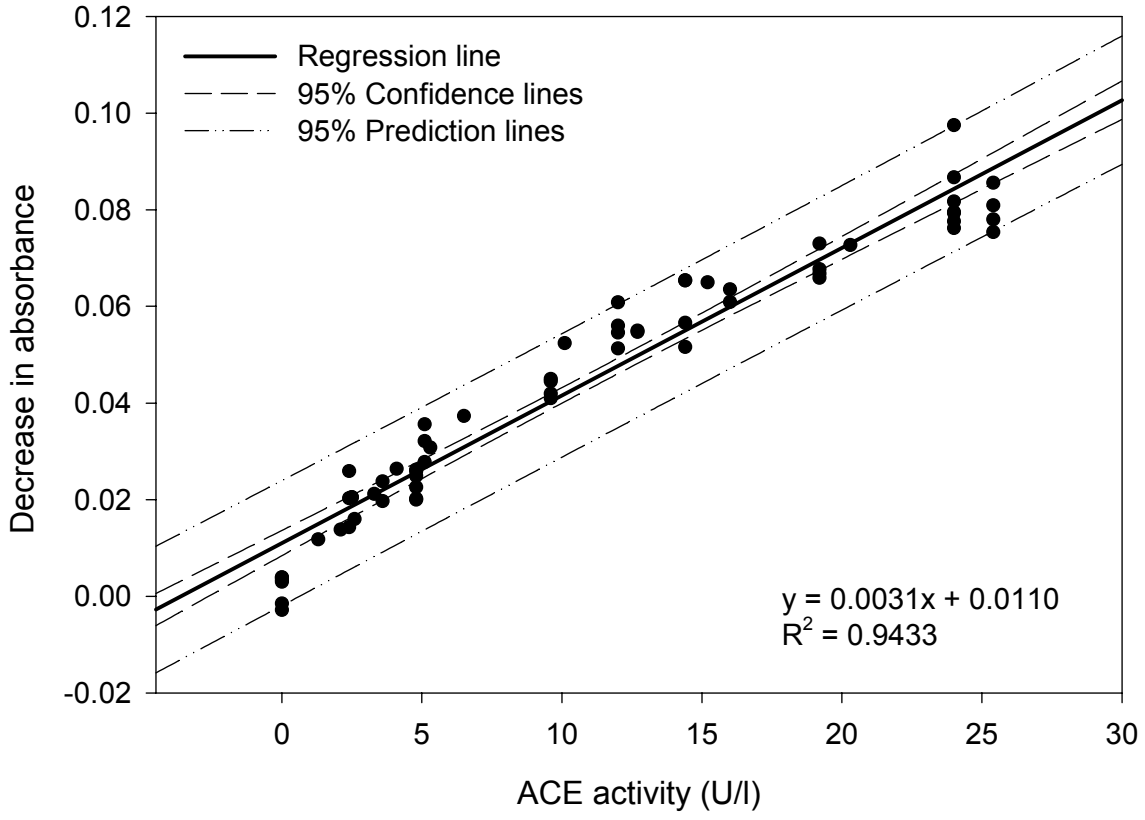


Figure 1. Standard curve for the ACE control-E, which links the decrease in absorbance over 5 min at 340 nm with the ACE activity in the reaction mixture (U/l) (n = 62). The 95% confidence and prediction lines of the standard curve are also shown. This standard curve was used in all assays.

Diagnostic assay transformed into an ACE inhibition assay: assay 1

Since in assay 1 only 100 µl ACE control-E enzyme was added, a maximum decrease in absorbance of 0.038 could be obtained for the ACE control-E during the 5 min measurement period (Figure 2). This corresponded to an ACE activity of 8.7 U/l. Taking the detection limit into account, this means that the reduction of the ACE activity to a value lower than 33% cannot be distinguished significantly from a reduction to 0% ($p = 0.003$).

The decrease in absorbance for a given ACE concentration occurred linearly in time during the 5 min measurement. Extending the measurement period to 11 min increased the decrease in absorbance, and thus the sensitivity, but the linearity of the decrease was not maintained: the decrease in absorbance during the last 6 min was on average 20% lower than expected from the linearity during the first 5 min of the measurement. Therefore it was decided to measure the ACE activity over a period of 5 min (data not shown).

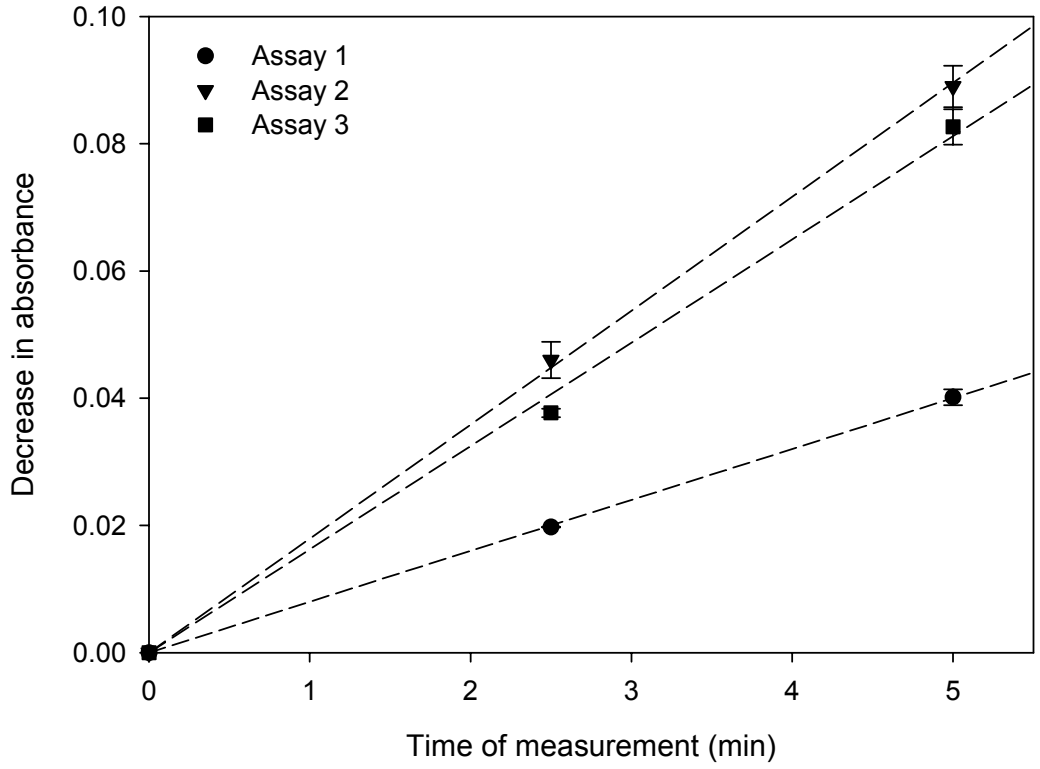


Figure 2. The decrease in absorbance over the 5 min measurement period for the three ACE inhibition assays (assay 1: 100 µl ACE control-E, assay 2: 300 µl ten times diluted rabbit lung acetone extract, assay 3: 300 µl ACE control-E) (n = 3).

In order to validate the ACE inhibition assay, captopril was added in different concentrations and ACE activity was determined. When the activities of the inhibited ACE solutions were plotted against the captopril concentration, a sigmoid curve was obtained (Figure 3). In the small concentration range of 10^{-10} M to 10^{-8} M there was a transition from

no to total inhibition of the ACE enzyme. Captopril is a very strong inhibitor, since its IC_{50} value was situated around 1.60 nM (Table 1).

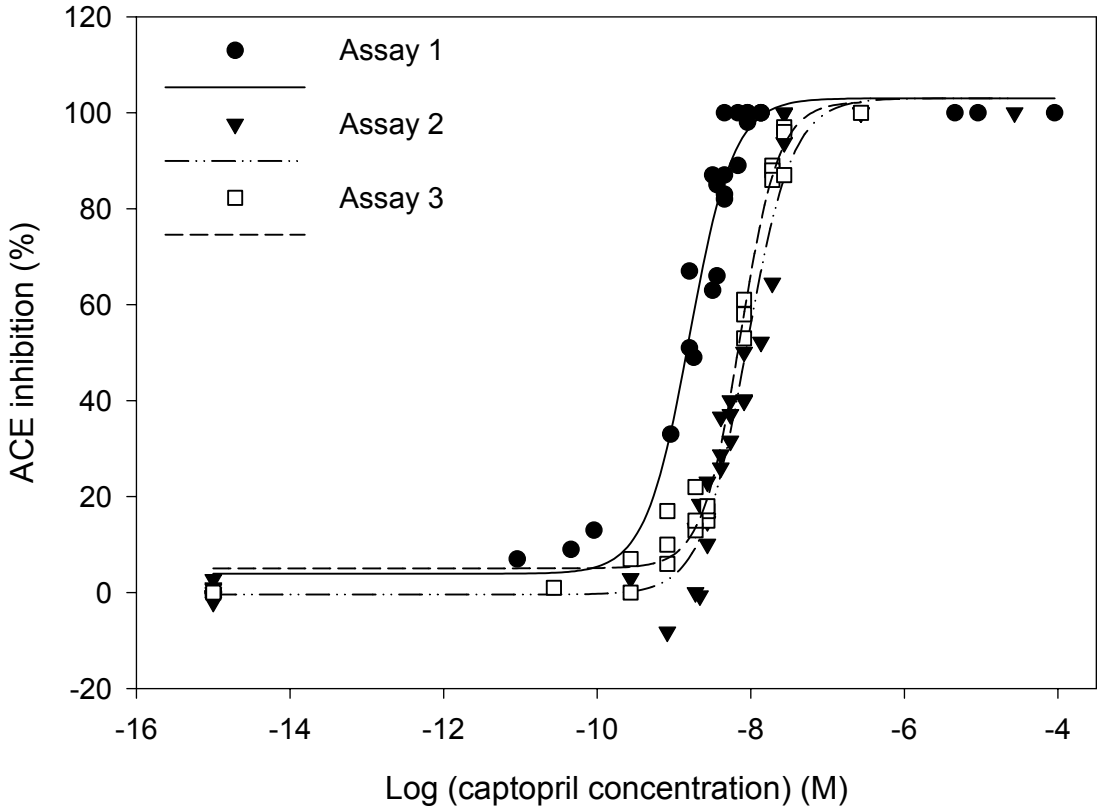


Figure 3. ACE inhibition (%) when captopril was added as inhibitory compound in assay 1 (●), assay 2 (▼) and assay 3 (□). Data were fitted by a 4 parametric logistic model (n_1 = 30, n_2 = 36, n_3 = 23).

ACE inhibition assay with rabbit lung acetone extract: assay 2

The ACE activity in the rabbit lung acetone extract is not known. Diluting the extract increased the linearity of the absorbance decrease over 5 min, while it reduced the sensitivity (Figure 4). Certain compounds in the extract also seemed to absorb at 340 nm. The ten times diluted rabbit lung acetone extract had about the same ACE activity as ACE control-E. The modified enzyme assay with ten times diluted rabbit lung acetone extract showed a good linear decrease in absorbance over the 5 min measurement period. The measurement range was also considerably increased compared to assay 1: an average decrease in absorbance of 0.09 was obtained, which slightly differed per batch rabbit lung acetone extract prepared (Figure 2). This corresponded with an ACE activity of 25.5 U/l in the standard curve of ACE control-E, which is approximately 3 times higher than the start ACE activity in ACE inhibition assay 1. Hence, a reduction of the ACE activity to 11% can still be distinguished from a reduction to 0% ($p = 0.003$). Batches of rabbit lung acetone extract were only used if the

decrease in absorbance for the blank was within the range of 0.07 to 0.11. Under these conditions no significant difference in IC_{50} for a given inhibitor was obtained with different batches.

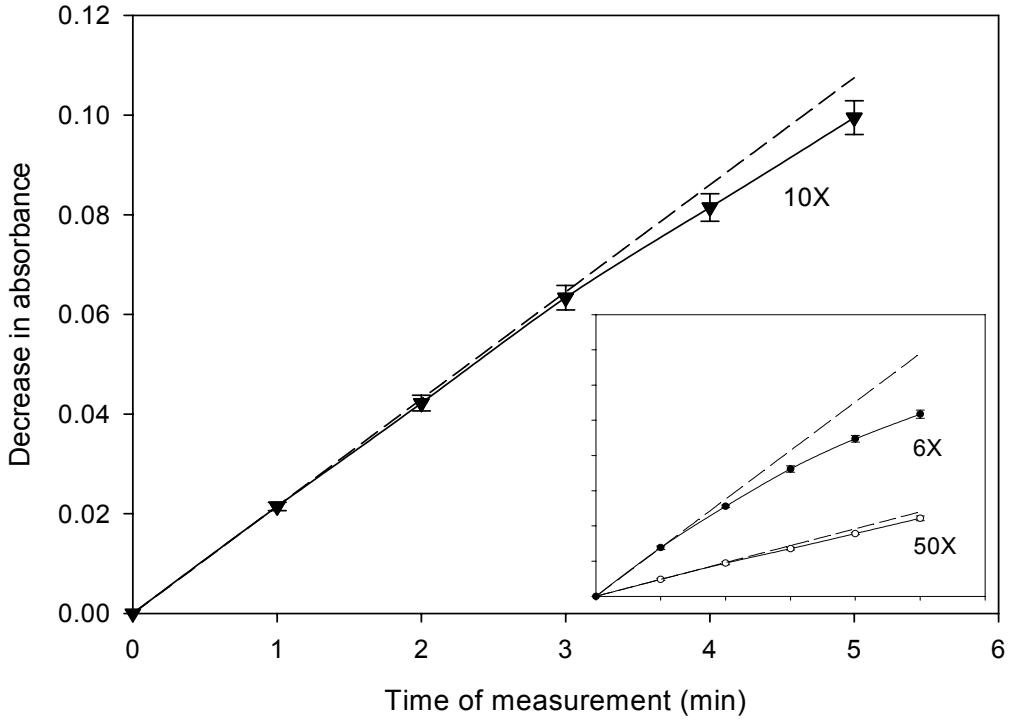


Figure 4. Deviation of the decrease in absorbance during the 5 min measurement from the initial rate of decrease (dashed line) for the ten times diluted rabbit lung acetone extract (inset: six and fifty times diluted rabbit lung acetone extract) (n = 3).

A spectrophotometric assay revealed the presence of about 606 mM chloride ions in the ACE reagent itself; the latter is diluted approximately a factor 5/11 in the assays. When pure elastase was used as enzyme source in the ACE inhibition assay, no significant decrease in absorbance was observed during the 5 min measurement at enzyme concentrations of 6.7 mg/ml and 0.67 mg/ml elastase.

Applying 300 µl pure angiotensin I converting enzyme (0.1 U Cushman & Cheung/ml) from rabbit lung in this ACE inhibition assay, revealed that 1 U of Sigma equals 1 U of Cushman & Cheung. In this respect, ACE inhibitory activities in different units can be compared.

With captopril a sigmoid curve was obtained similar to this of assay 1 (Figure 3, Table 1). In the concentration interval of 10^{-10} M to 10^{-7} M there was a change from 0 to 100% inhibition of the ACE enzyme. Here, captopril had an IC_{50} of 8.91 nM, which was significantly different from the one in assay 1 ($p < 0.001$). When enalapril, enalaprilat or the ACE inhibitory peptide Ala-Leu-Pro-Met-His-Ile-Arg were dosed in the ACE inhibition assay with

rabbit lung acetone extract, sigmoid inhibition curves were observed as well (Figure 5). Enalaprilat is the active antihypertensive drug, which was obtained by chemical hydrolysis from the prodrug enalapril. This conversion resulted in a decrease in the IC_{50} value from 1.56 µM to 4.96 nM, which corresponds to an augmentation of the ACE inhibitory activity with a factor 1000. The food derived ACE inhibitory peptide was less active, with an IC_{50} value of 32.5 µM (Table 1).

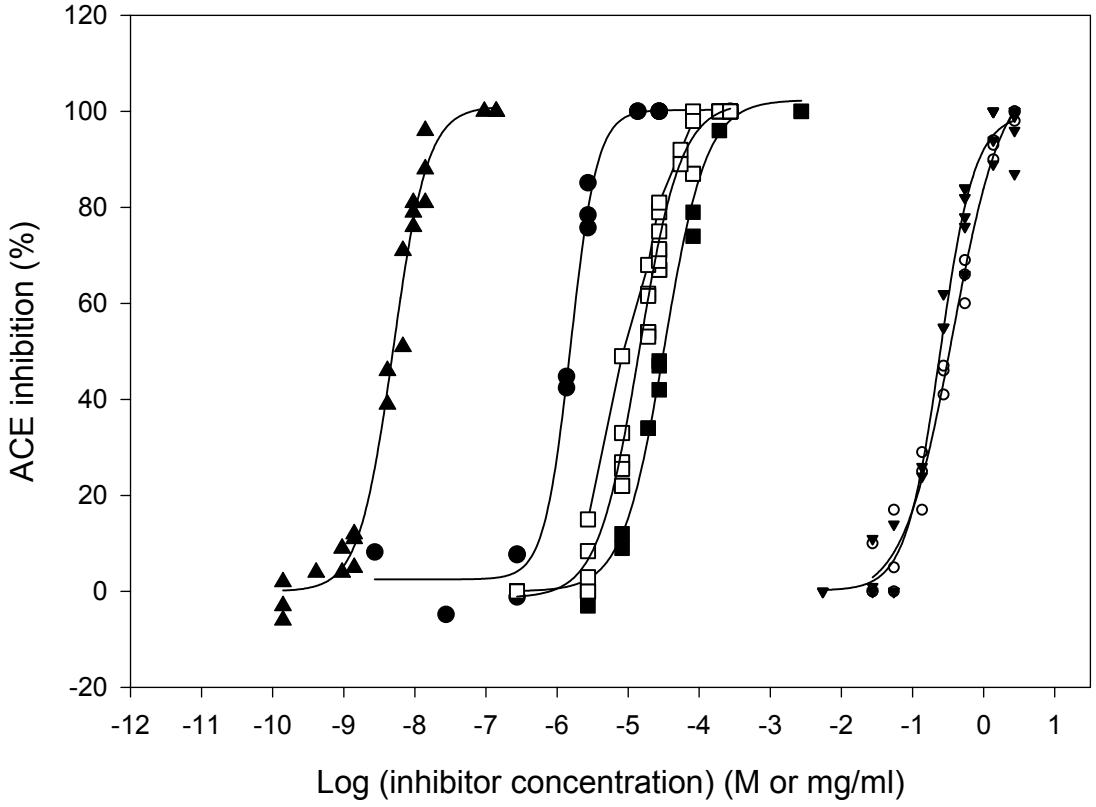


Figure 5. ACE inhibition (%) when enalaprilat (▲), enalapril (●) and Ala-Leu-Pro-Met-His-Ile-Arg (■), whey digest (▼) an pea digest (○) were used as inhibitory compound in assay 2. Ala-Leu-Pro-Met-His-Ile-Arg in assay 3 (□) is also plotted. Data were fitted by a 4 parametric logistic model ($n_{enalapril}$ = 16, $n_{enalaprilat}$ = 22, $n_{peptide}$ = 22, $n_{peptide\text{-}assay3}$ = 36, n_{whey} = 31, n_{pea} = 21).

Whey and pea protein isolate were subjected to a digestion with trypsin (E/S = 1/250 (w/w)) for 2 h at 37°C and pH 7. Before digestion, the measured ACE inhibitory activity in whey and pea protein was 9 ± 4% and 35 ± 4% respectively. After digestion, these values increased to 97 ± 2% and 99 ± 1% for whey and pea protein respectively. When fitting a 4 parametric logistic model to dose-activity data, IC_{50} values of 0.24 mg/ml for whey and 0.37 mg/ml for pea protein digest were observed ($p < 0.01$) (Figure 5, Table 1).

ACE inhibition assay with pure ACE from porcine kidney: assay 3

ACE control-E has an assigned ACE activity, which differs slightly per lot product. During our experiments, this value ranged from 88 to 102 U/l at 37°C, which corresponds with a concentration in the assay from 24 to 27.8 U/l and an absorbance decrease from 0.085 to 0.097. This is very similar to assay 2 (Figure 2).

Table 1. Parameters of the fitted 4 parametric logistic dose-activity curves for the following inhibitors: captopril, enalapril, enalaprilat, the ACE inhibitory peptide Ala-Leu-Pro-Met-His-Ile-Arg and the tryptic digests of whey and pea protein in assay 1[1], assay 2[2] and assay 3[3].

	Min (%)	Max (%)	Log IC_{50} (M)	Hill slope	R^2	N
Captopril[1]	3.9 ± 2.7	103.0 ± 3.3	-8.80 ± 0.05	1.5 ± 0.2	0.97	30
Captopril[2]	-0.4 ± 2.4	103.0 ± 3.7	-8.05 ± 0.04***	1.5 ± 0.2	0.96	36
Captopril[3]	5.0 ± 1.9	102.3 ± 3.7	-8.13 ± 0.04	1.7 ± 0.2	0.99	23
Enalapril[2]	2.5 ± 1.9	100.3 ± 1.7	-5.81 ± 0.02	2.3 ± 0.3	0.99	16
Enalaprilat[2]	0.0 ± 2.5	101.0 ± 3.0	-8.30 ± 0.03	1.8 ± 0.2	0.99	22
Peptide[2]	0.0 ± 1.5	102.4 ± 1.8	-4.49 ± 0.02	1.5 ± 0.1	0.99	22
Peptide[3]	-1.4 ± 3.0	101.8 ± 2.6	-4.84 ± 0.03***	1.5 ± 0.2	0.99	36
Whey digest[2]	0.2 ± 2.6	99.4 ± 2.3	*-0.61 ± 0.03*	1.8 ± 0.2	0.99	31
Pea digest[2]	-1.3 ± 4.2	108.6 ± 6.3	*-0.43 ± 0.05***	1.2 ± 0.2	0.99	21

Values are expressed as mean ± standard error.
Significant differences (*p < 0.05, **p < 0.01, ***p < 0.001) verified by t-tests between captopril[1] and [2], and captopril[2] and [3], peptide[2] and [3] and whey and pea digest respectively.
IC_{50} of whey and pea digest is expressed in mg/ml.

When compared to the curve of assay 2, inhibition with captopril showed an almost identical sigmoid curve (Figure 3). An IC_{50} value of 7.41 nM was calculated, which was not significantly different from the one obtained in assay 2 (Table 1). Ala-Leu-Pro-Met-His-Ile-Arg displayed an IC_{50} value of 14.5 µM in assay 3, which was significantly different from the one in assay 2 ($p < 0.001$) (Table 1, Figure 5). The repeatability of the log IC_{50} of this lactokinin, expressed as variation coefficient (standard deviation over mean), was 7.5% in assay 3 (n = 3). The reproducibility, expressed as variation coefficient, amounted to 3.6% (n = 3).

Furthermore, the peptide Gly-Gly-Gly at a concentration of 2.73 mg/ml in the reaction mixture had an ACE inhibitory activity of 97 ± 3%.

Table 1 shows a good fit to the 4 parametric logistic model for all inhibitory compounds in all assays: regression coefficients were higher than 95%. In all cases the ACE inhibitory activity increased from 0 to 100% in function of the concentration. The hill slope was always higher than 1.2, which corresponds to an angle of more than 50°.

DISCUSSION

Most ACE inhibitory drugs and peptides inhibit the ACE enzyme in a competitive way (Meisel, 1997a; Weisser and Schloos, 1991). Above a given concentration a saturation effect appears: ACE inhibitory activity is then 100% and independent of the inhibitor concentration. Below a given concentration ACE inhibitory activity is absent. Between 0 and 100% the ACE inhibitory activity changes according to a sigmoid curve in function of the logarithm of the concentration. A 4 parametric logistic model can fit the data points, from which the IC_{50} value, the concentration of inhibitor that inhibits the ACE enzyme for 50%, is calculated. This model efficiently described the ACE inhibitory activity in function of the inhibitor concentration, regardless of assays or inhibitory compounds. The calculated hill slope is an indication of the rate of change of the sigmoid curve. For all inhibitory compounds a steep slope was observed.

Measuring the ACE activity with FAPGG as a substrate is a sensitive method for substrate concentrations above the Michaelis-Menten constant K_m ($3 .10^{-4}$ M), to give classical zero-order kinetics, and below K_m, to give first-order kinetics (Holmquist *et al.*, 1979; Ronca-Testoni, 1983). In our assays the substrate concentration was $4.6 .10^{-4}$ M, hence higher than K_m, for which the reaction obeys zero order kinetics in the substrate concentration under steady state conditions. The reaction rate, which is expressed as the ACE inhibitory activity, is then independent of the substrate concentration and approaches v_{max} in the enzyme saturation kinetics, which is directly proportional to the enzyme concentration. Under these conditions substrate hydrolysis occurs linearly with time over at least 15% of total hydrolysis (Holmquist *et al.*, 1979). Provided that the absorbance decreases linearly with time during the 5 min measurement period, the decrease in absorbance is a measure for the reaction rate or the enzyme activity ($\mu mol.l^{-1}.min^{-1}$) (Weisser and Schloos, 1991). Furthermore, FAPGG is the substrate of choice since it exhibits one of the highest activities with ACE. Its kinetic parameters are better than those of Hip-His-Leu, the most commonly used ACE substrate (Cushman and Cheung, 1971; Meisel *et al.*, 1997a; Mullally *et al.*, 1997a;

Nakamura *et al.*, 1995a; Pihlanto-Leppällä *et al.*, 1998). FAPGG has a K_m = 3 .10^{-4} M and k_{cat} = 19 000 min^{-1} which compare favourably with values of 2.4 .10^{-3} M and 15 600 min^{-1} respectively for Hip-His-Leu (Bunning, 1983; Bunning *et al.*, 1983; Holmquist *et al.*, 1979). The diagnostic Sigma reagent kit is already validated for measuring ACE activity. Wong and Kinniburgh (1987) have shown that the reaction is linear up to 160 U/l ACE, which corresponds with a concentration in assays 2 and 3 of 43.6 U/l.

The sensitivity of the diagnostic ACE inhibition assay (assay 1) could not be increased by prolonging the incubation time, because zero order Michaelis-Menten kinetics were no longer applicable under these conditions. The switchover to the ACE inhibition assay with rabbit lung acetone extract (dilution 1/10) and applying the appropriate enzyme-substrate ratio (assay 2), increased the useful measurement range of the assay three times. Since in assay 2 rabbit lung acetone extract replaced the pure porcine ACE-control E, it is less expensive. Moreover, less inhibitor was needed in this assay. Cushman and Cheung (1971) stated an optimal ACE activity of the rabbit lung acetone extract at pH 8.1-8.3 and in the presence of 300 mM NaCl. Chloride ions are necessary for the activity of the enzyme. In our reaction mixtures a chloride ion concentration of about 275 mM was present, which approximates the optimal chloride ion concentration. The concentration of chloride ions in the reagent itself is in agreement with the value of 651 mM, reported by Wong and Kinniburgh (1987). Holmquist *et al.* (1979) mentioned with furanacryloyl tripeptides as substrate and purified ACE from rabbit lung acetone powder a maximal hydrolysis rate at pH 7.5. Since in the Sigma diagnostic assay the ACE reagent is buffered at pH 8.2, the reaction in our experiments also took place at this pH. According to Cushman and Cheung (1971) this is also the pH where the rabbit lung acetone powder extract shows optimal ACE activity. Rabbit lung acetone powder is sold by Sigma as a source of angiotensin converting enzyme and elastase, a non-specific protease. Pure elastase was used in assay 2 as enzyme source to verify that elastase was not able to cleave the substrate FAPGG. As this was not the case, rabbit lung acetone powder is specific enough to search for ACE inhibitory activity with FAPGG as substrate.

Later batches of the rabbit lung acetone extract failed to give a satisfactory absorbance decrease of at least 0.07. Therefore, assay 2 was modified by using ACE control-E as enzyme. This decreased the cost-efficiency of the assay, but on the other hand resulted in a more standardised assay.

The active inhibitory group in captopril is a sulfhydryl (SH) group that chelates the zinc ion contained in the active site of the ACE enzyme (Bunning, 1983; Bünning, 1987; Cushman *et al.*, 1987; Shapiro and Riordan, 1984). The obtained IC_{50} values for captopril, 1.60 .10^{-9} M in assay 1, 8.91 .10^{-9} M in assay 2 and 7.41 .10^{-9} M in assay 3, correspond well with literature,

where values from 7.5 $.10^{-10}$ M to 2.2 $.10^{-8}$ M are mentioned (Cushman *et al.*, 1987; Cushman *et al.*, 1989; Elbl and Wagner, 1991; Fujita and Yoshikawa, 1999; Hiwada *et al.*, 1990; Ino *et al.*, 1989; Mullally *et al.*, 1997a; Williams *et al.*, 1993). The difference in IC_{50} for captopril in the Sigma diagnostic assay (assay 1) and the ACE inhibition assay with rabbit lung acetone extract (assay 2) can be explained by the different test conditions, a different origin of the ACE enzyme and the complex composition of the rabbit lung acetone extract. However, captopril demonstrated in assay 3 with ACE from porcine kidney a similar IC_{50} as in assay 2 with rabbit lung acetone extract. Ariyoshi (1993) emphasised that any ACE inhibitor may act differently against ACE obtained from different sources. Some researchers include another blank, which contains the inhibitory compound, but no ACE enzyme, resulting in a different calculation of the ACE inhibitory activity (Nakamura *et al.*, 1995a). Furthermore, when comparing to literature data, one has to consider that different substrates are used in the determination of ACE inhibitory activity, which result in different IC_{50} values (Brooks *et al.*, 1990; Weisser and Schloos, 1991).

Enalaprilat is dosed as prodrug, enalapril, to improve the absorption after oral intake. In enalapril the active inhibitory group, a COOH-group, is esterified with an alcohol and the release of the active compound enalaprilat occurs after absorption by esterases in the plasma (Chevillard *et al.*, 1994). Enalapril on its own exerts a certain inhibitory capacity that is about thousand times less potent than that of enalaprilat. The IC_{50} value of enalapril, 1.56 $.10^{-6}$ M, reported here, corresponds well with literature data, where values between 2.1 $.10^{-7}$ M and 7 $.10^{-5}$ M are mentioned (Chevillard *et al.*, 1994; Hiwada *et al.*, 1990; Udupa and Rao, 1997). Enalaprilat further confirmed the validity of our assay. The observed IC_{50} of 4.96 $.10^{-9}$ M falls in the range of 1 $.10^{-10}$ M to 6 $.10^{-9}$ M found in literature (Allan *et al.*, 1987; Hiwada *et al.*, 1990; Inada *et al.*, 1988; Tillman and Moore, 1989; Weisser and Schloos, 1991). This may indicate that the assumption of a complete conversion of enalapril to enalaprilat during alkaline hydrolysis was correct.

Not only ACE inhibitory drugs, but also the food derived ACE inhibitory peptide Ala-Leu-Pro-Met-His-Ile-Arg, inhibited the ACE enzyme in assay 2 and 3 in a similar way as reported elsewhere. The IC_{50} of 32.5 µM for the peptide in assay 2 corroborates well with the value of 42.6 µM found by Mullally *et al.* (1997b). In assay 3, a slightly higher ACE inhibitory activity (IC_{50} = 14.5 µM) for this ACE inhibitory peptide was obtained. In fact, the ACE inhibition assay with pure ACE (assay 3) generally gave slightly higher ACE inhibitory activities compared to assay 2. This may be due to an increased sensitivity as a result of the purity of the enzyme source.

Trypsin digestion of whey and pea protein produced ACE inhibitory activity in assay 2. Before enzyme addition a relatively high ACE inhibitory activity was detected in the pea

protein solution, which can be due to some acid or basic hydrolysis during pH setting. IC_{50} values for the hydrolysates were about 0.3 mg/ml, which corresponds to 0.3 mM assuming an average molecular weight of 1000 Da for the ACE inhibitory peptides. There were significant differences between the fitted curves of the whey and pea digest, indicating different ACE inhibitory peptides. Mullally *et al.* (1997a) obtained ACE inhibitions of 80-90% and IC_{50} values ranging from 130-201 mg/l for ultrafiltrated tryptic digests of β-lactoglobulin and whey protein concentrate. Pihlanto-Leppällä *et al.* (1998) found about the same ACE inhibitory activity in a peptic and tryptic digest of whey: IC_{50} values ranging from 80-314 mg/l for different chromatography fractions. An isolated peptide from this digest, β-lactoglobulin f(9-14), released by trypsin, had an IC_{50} value of 580 µM. In a later article, where α-lactalbumin and β-lactoglobulin are digested by trypsin, an IC_{50} value of 345 respectively 457 mg/l is mentioned for the total hydrolysate, while after fractionation higher ACE inhibitory activities are observed (Pihlanto-Leppälä *et al.*, 2000). These values are similar to the ones for the tryptic pea and whey protein hydrolysates reported here.

Gly-Gly-Gly showed potent ACE inhibitory activity in ACE inhibition assay 3, however, this peptide is known to be a substrate for ACE (Ohkubo *et al.*, 1994). As ACE prefers this small tripeptide substrate over FAPGG, no decrease in absorbance is observed. This is the limitation of all spectrophotomectric ACE inhibition assays. A distinction between a real inhibitor and a substrate can be made by pre-incubation with ACE, follow-up of the degradation by HPLC and measurement of the ACE inhibitory activity before and after (Fujita *et al.*, 2000). Otherwise, an overestimation of the ACE inhibitory activity is obtained *in vitro*. Though, few authors in literature take this fact into account and prefer not to complicate the assay. Our peptide mixtures also hamper HPLC analysis. In addition, as heat treatment is necessary to inactivate the ACE from the pre-incubation before ACE inhibition can be measured, degradation of the peptides is risked.

In conclusion, two ACE inhibition assays, one with rabbit lung acetone extract and another with pure ACE from porcine kidney as ACE source, were demonstrated to be relative rapid and reliable tools for the screening of food derived bioactive peptides.

ACKNOWLEDGEMENTS

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CHAPTER 3

STABILITY OF THE LACTOKININ ALA-LEU-PRO-MET-HIS-ILE-ARG IN THE ORAL DELIVERY ROUTE *IN VITRO*

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Chapter 3

Stability of the lactokinin Ala-Leu-Pro-Met-His-Ile-Arg in the oral delivery route in vitro

ABSTRACT

ACE inhibitory peptides derived from food proteins during food processing or gastrointestinal digestion, could function as efficient agents in treating and preventing hypertension. However, in order to exert an antihypertensive effect by inhibition of the ACE enzyme, they have to reach the systemic circulation. The aim of this research was to assess if the known ACE inhibitory peptide Ala-Leu-Pro-Met-His-Ile-Arg, derived from a tryptic digest of β-lactoglobulin, could resist digestion by gastrointestinal proteases and peptidases from the intestinal brush border and enterocytes. In addition, it was investigated if this peptide could cross a Caco-2 Bbe cell monolayer mounted in an Ussing chamber and reach the serosal side intact. The heptapeptide was partially degraded during digestion with α-chymotrypsin; at the end of 4.5 h *in vitro* gastrointestinal digestion, more than half of the initial peptide concentration could still be recovered. The lactokinin was rapidly broken down by rat intestinal brush border peptidases, while almost no degradation was observed after incubation with Caco-2 homogenates. In the transport experiment, samples of the mucosal compartment showed high ACE inhibitory activity 10 min after the addition of 1 mM of the heptapeptide. No or only little ACE inhibitory activity was detected in the serosal compartment, although a substantial ACE inhibitory activity was registered after threefold concentration. Concomitantly, HPLC and MS clearly showed the presence of Ala-Leu-Pro-Met-His-Ile-Arg in the mucosal compartment, whereas in the serosal compartment only MS was able to detect the heptapeptide. Hence, under the observed experimental conditions, the ACE inhibitory peptide Ala-Leu-Pro-Met-His-Ile-Arg was transported intact through the Caco-2 Bbe cell monolayer, although at concentrations too low to exert an ACE inhibitory activity *in vitro*. In conclusion, it seems that the degradation by intestinal brush border peptidases constitutes the major barrier in the oral delivery route of this lactokinin.

Keywords: ACE inhibitory peptide, lactokinin, gastrointestinal digestion, intestinal transport, Caco-2, whey protein, Ussing Chamber

INTRODUCTION

Bioactive peptides are food derived peptides that have, besides their nutritional, also a functional effect in the human body: they can behave as regulatory components with a hormone like activity (Clare and Swaisgood, 2000). In this respect, stability to human proteases and intestinal transport of active peptides are often prerequisites.

ACE inhibitory peptides are bioactive peptides with possible blood pressure lowering effects *in vivo*. By inhibiting the angiotensin I converting enzyme (ACE; EC 3.4.15.1), the concentration of the vasoconstrictor angiotensin II decreases, while the concentration of the vasodilator bradykinin increases. This results in an antihypertensive effect (Lüscher and Yang, 1993). ACE inhibitory peptides have already been found in several food protein hydrolysates and ferments (Dziuba *et al.*, 1999a; Yamamoto, 1997) and have been shown to lower the blood pressure in spontaneously hypertensive rats (SHR) and hypertensive patients (Hata *et al.*, 1996; Yoshii *et al.*, 2001). Therefore, there is a growing interest in using these bioactive peptides as efficient agents in treating and preventing hypertension.

ACE inhibitory peptides can only exert an antihypertensive effect if they reach the bloodstream intact after oral administration. Therefore, they have to show some resistance to gastrointestinal proteases and brush border peptidases and they have to be absorbed through the intestinal wall with preservation of their physiological activity. Hence, either the orally administered ACE inhibitory peptide or an active metabolite has to enter the systemic circulation. The peptide in the present study, Ala-Leu-Pro-Met-His-Ile-Arg, is a lactokinin, a whey protein derived ACE inhibitory peptide, corresponding to a tryptic fragment of bovine β-lactoglobulin, with an IC_{50} value of 42.6 µM (Mullally *et al.*, 1997b). According to this study, this peptide is resistant to further digestion with pepsin and is only hydrolysed to a very low extent by α-chymotrypsin. Thus, this peptide may reach the brush border membrane intact. Passage of intact peptides across the intestinal mucosa may occur via both paracellular and transcellular routes (Grimble and Backwell, 1998). It is generally accepted that di- and tripeptides are transported, because these are the substrates for the intestinal peptide transporter PEPT1 (Grimble, 2000; Yang *et al.*, 1999). There is also evidence that larger peptides may be absorbed through the small intestine (Chabance *et al.*, 1998; Gardner, 1984; Roberts *et al.*, 1999), although it remains to be established to which extent this occurs and what the transport mechanism is.

In this study, the temperature-dependent chemical degradation of Ala-Leu-Pro-Met-His-Ile-Arg was studied. Subsequently, the peptide was exposed to an *in vitro* gastrointestinal digestion with pepsin (A, EC 3.4.23.1), trypsin (EC 3.4.21.4) and α-chymotrypsin (EC

3.4.21.1). Furthermore, the resistance of the lactokinin to degradation by intestinal brush border and intracellular peptidases was tested. In an Ussing chamber experiment we investigated if the ACE inhibitory heptapeptide could cross a monolayer formed by a Caco-2 cell clone, the Caco-2 Bbe (morphologically homogeneous, brush border expressing cells) cell monolayer, which is a good model for the small intestinal epithelium (Peterson and Mooseker, 1992; Wilson *et al.*, 1990). The presence of the peptide was measured by an *in vitro* ACE inhibition assay and by HPLC and MS. Preliminary experiments assessed the antihypertensive activity of the lactokinin after intravenous administration in SHR.

MATERIALS AND METHODS

Products

The ACE inhibitory peptide Ala-Leu-Pro-Met-His-Ile-Arg (836.5 g/mol) was synthesised by solid phase peptide chemistry and FMOC protection and evaluated by HPLC-MS (purity: $batch_1$ = 84.37%, $batch_2$ = 86.99%, $batch_3$ = 80.97% (w/w)) at the Biotechnology Center of the University of Illinois (Urbana-Champaign, IL, USA).

Pepsin (P 6887), trypsin (T 1426) and α-chymotrypsin (C 4129) were purchased from Sigma-Aldrich, St.-Louis, MO, USA.

Krebs buffer was composed of 111.9 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 1.6 mM Na_2HPO_4, 25 mM $NaHCO_3$, 4.9 mM Na-(L-)glutamate, 4.9 mM Na-pyruvate, 5.4 mM Na_2-fumarate and 11.5 mM glucose at pH 7.4. Transport buffer contained Hanks' Balanced Salt Solution supplemented with HEPES (both from Invitrogen Life Technologies, Carlsbad, CA, USA) and D-glucose (Sigma-Aldrich, G 7021) at a final concentration of 10 mM and 25 mM, respectively at pH 7.4. Modified Krebs buffer consisted of 140 mM Na^+, 119.8 mM Cl^-, 25 mM HCO_3^-, 1.2 mM Mg^{2+}, 1.2 mM Ca^{2+}, 4.8 mM K^+, 2.4 mM HPO_4^{2-} and 0.4 mM $H_2PO_4^-$ at pH 7.4.

Non-specified products were analytical grade from VWR International (Zaventem, Belgium).

Stability and digestion experiments

Chemical stability

The peptide, dissolved at a concentration of 60 mg/l (72 µM) in Krebs buffer (pH 7.4) was incubated at 4°C, 25°C (room temperature) and 37°C, respectively. The degradation was monitored by RP-HPLC.

In vitro gastrointestinal digestion

A 125 µM (105 mg/l) Ala-Leu-Pro-Met-His-Ile-Arg solution in MilliQ water (Millipore, Bedford, MA, USA) was subjected to a digestion by pepsin for 2 h at pH 2 (E/S = 1/200 (w/w)), followed by a hydrolysis by trypsin and α-chymotrypsin for 2.5 h at pH 7.5 (E/S = 1/200 (w/w)) at 37°C. The concentration of the peptide during incubation was determined by RP-HPLC and the ACE inhibitory activity was analysed by the ACE inhibition assay (assay 3, Chapter 1). This experiment was performed twice. As a control, a pepsin solution and a solution containing pepsin, trypsin and α-chymotrypsin, at a concentration ten times the one in the digestion experiment, were also analysed by RP-HPLC. The lactokinin was theoretically cleaved by the program Peptide Cutter at www.expasy.ch. The degradation products of the peptide were determined by Matrix Assisted Laser Desorption / Ionization - Time Of Flight (MALDI-TOF) spectrometry (Bruker Reflex III, Bruker Daltonik, Bremen, Germany) (Gevaert *et al.*, 2001).

Stability towards peptidases from the intestinal brush border and enterocytes

The peptide was added to the buffer solution that originated from the mucosal and serosal chamber respectively of an Ussing chamber transport experiment with rat intestinal tissue. Both buffer solutions, which contained released enzymes from the rat intestinal tissue in Krebs buffer, were kept for 16 days at -80°C. The initial peptide concentration was about 30 mg/l (36 µM). The solutions were incubated for 4 h at 37°C.

Moreover, 100 µM Ala-Leu-Pro-Met-His-Ile-Arg dissolved in transport buffer (pH 7.4), was incubated in the presence of Caco-2 homogenates in an E/S of 1/6 (w/w) for 3 h at 37°C. These Caco-2 homogenates were prepared as described by Augustijns *et al.* (1998). In brief, freshly scraped Caco-2 cell monolayers grown in a 75 cm^2 flask were homogenised in 5 ml transport buffer using a cell disrupter (Branson Sonifier B15, Branson Ultrasonics, Frankfurt, Germany) for 10 s at 4°C. After centrifugation of the crude Caco-2 homogenates at 14 000 g for 5 min, the supernatants were harvested and the protein content was determined by the

method of Lowry (Lowry *et al.*, 1951). The Caco-2 homogenates were kept for 6 months at -80°C.

The concentration of the peptide at different time intervals, as well as the mucosal and serosal buffer solution, and the Caco-2 homogenate in transport buffer, were analysed by RP-HPLC.

HPLC

The lactokinin was analysed by reversed-phase HPLC on a Prosphere 300 Å C_{18} column (250 x 4.6 mm, 5 µm) (Alltech Associates, Deerfield, IL, USA) and a Dionex (Sunnyvale, CA, USA) HPLC with an autosampler ASI-100, pump series P580, STH585 column oven, UV-VIS detector UVD340S operating at 210 nm and Chromeleon 6.0 software. Elution was at 25°C with a flow rate of 1 ml/min. A linear gradient was applied from 90% solvent A (H_2O + 0.1% (w/v) TFA) to 50% solvent B (acetonitrile + 0.085 % (w/v) TFA) in 30 min, again to 90% solvent A in the next 20 min and remaining at 90% solvent A during the last 10 min. Quantitative determination of Ala-Leu-Pro-Met-His-Ile-Arg was done by injection of 100 µl sample and using an external standard ranging from 0.01 to 0.2 mM. The peptide eluted at a retention time of 13.8-14.1 min with a detection limit of 5 µM. The precision of the injection of 0.1 mM of the lactokinin had a variation coefficient (standard deviation over mean) of 3.5% (n = 10).

Transport experiments

Cell culture

Caco-2 Bbe (Peterson and Mooseker, 1992) cells were routinely grown in T-75 cell culture flasks in Dulbecco's Modified Eagle Medium (DMEM), containing 10% (v/v) fetal bovine serum, 0.25 mg/l fungizone and 0.5% (v/v) penicillin-streptomycin, supplemented with non-essential amino acids (all from Invitrogen Life Technologies) and 10 mg/l transferrin (Sigma-Aldrich, T 5391), in an atmosphere of 20% O_2 and 5% CO_2 at 37°C. For the Ussing Chamber experiments, 10^5 cells/well (passage 95) were inoculated in Costar Brand Snapwell culture plates (3 µm pore diameter, 12 mm diameter, Costar, Cambridge, MA, USA) and maintained for 23 days with daily medium refreshment.

Electrophysiological studies

The Snapwell inserts with the Caco-2 Bbe monolayer were mounted in modified Ussing chambers (Physiological Instruments Inc., San Diego, CA, USA) (Figure 1) with an exposed

area of 1.13 cm^2 (Kles *et al.*, 2001). Cells were bathed on each side with 4 ml oxygenated (95% O_2/5% CO_2) modified Krebs buffer (pH 7.4) maintained at 37°C and short circuited after appropriate correction for fluid and system resistance, using dual channel voltage/current clamps (VCC MC2, Physiological Instruments) connected with a computer interface (Acquire & Analyze Software, Physiological Instruments).

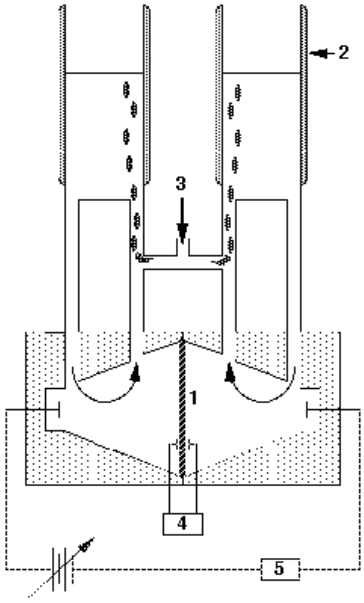

Figure 1. Schematic overview of an Ussing chamber set-up. Indicated are the intestinal tissue or cell insert (1), water-jackets (2), gas-inlet (3), potential difference (4) and short-circuit current (5) measurement.

Serosal solutions were supplemented with 10 mM glucose while mucosal solutions were supplemented with 10 mM mannitol for osmotic balance. The transepithelial potential difference (P_D) and the current required for nullifying the spontaneous transepithelial potential difference (I_c) were monitored. At P_D = 0 mV, respective short-circuit currents (I_{sc}) were calculated. Periodic measurements of potential difference and current deflections induced by bipolar pulses of 1 s duration and 66 µA amplitude, served to calculate the transepithelial electrical resistance (R) based on Ohmic relationship. Ala-Leu-Pro-Met-His-Ile-Arg was added to the mucosal bathing solution at a final concentration of 1 mM while equimolar amounts of mannitol were added to the serosal side (n = 6). One Ussing chamber served as a control. After 10 min, samples were taken from both the mucosal and serosal side for the ACE inhibition assay and HPLC-MS. Samples were freeze-dried and redissolved prior to analysis. In the Ussing chambers, samples were replaced by equal volumes of buffer and 10 mM L-glutamine was added to the mucosal compartments together with 10 mM mannitol to the serosal compartments, to check for an I_{sc} increase indicating cell monolayer

viability and integrity during the incubation period. Responses to substrate supplementation were expressed as differences between basal value and maximal response (ΔI_{sc}).

ACE inhibitory activity and ACE activity

Lyophilised samples were redissolved to the original volume in demineralised water, exept for the concentrated analysis on two Ussing chambers, where the lyophilised powder was dissolved in a volume threefold smaller than the original one. The ACE inhibitory activity was measured by the ACE inhibition assay with ten times diluted rabbit lung acetone extract (assay 2 in Chapter 2) (Vermeirssen *et al.*, 2002b). To assess the ACE activity in the control, which accounted for the enzyme released by the cell monolayer in the buffer solution, 300 µl of mucosal and serosal bathing solution respectively, was used as enzyme source in the ACE inhibition assay. Demineralised water was added instead of an inhibitory compound.

HPLC and MS

The bathing solutions of two Ussing chambers in which the heptapeptide was supplemented, were analysed by HPLC and MS. Reversed-phase HPLC analysis was performed on a Beckman C_{18} Ultrasphere column (150 x 4.6 mm, 5 µm) and a Beckman HPLC equipment model 126 (Fullerton, CA, USA) with an UV detector operating at 215 nm. A 40 µl aliquot of 1 mg/ml lyophilized powder in 0.1% (w/v) TFA in water was loaded onto the column and eluted at a flow rate of 0.5 ml/min with a linear gradient from 100% solvent A (0.06% (w/v) TFA in water) to 80% solvent B (0.052% (w/v) TFA in acetonitrile) in 60 min at 25°C. Samples were also analysed by MALDI-TOF spectrometry on a Voyager-DE STR (Applied Biosystems, Foster City, CA, USA) using positive reflectron mode with 100 ns delayed extraction, an accelerating potential of 20 kV (76% grid voltage, 0% guide wire voltage) and external calibration to convert the time-of-flight to mass. Prior to analysis, the samples were acidified with aequous TFA to pH < 4 and a final TFA concentration between 0.1%-1%, subsequently concentrated and desalted using C_{18} ZipTip (Millipore, Bedford, MA, USA) and finally crystallised with α-hydroxycinnamic acid (Sigma-Aldrich). The detection limit of the MALDI-TOF spectrometer was about 1 µg/l.

Antihypertensive activity

The lactokinin was injected intravenously at a dose of 5 mg/kg BW in the femoral vein of Spontaneously Hypertensive Rats (SHR) (n = 2). The complete procedure and the measurement of the Mean Arterial Blood Pressure (MAP) are described in Chapter 7.

RESULTS

Chemical stability of Ala-Leu-Pro-Met-His-Ile-Arg

The temperature-dependent degradation of the lactokinin is illustrated in Figure 2. After 24 h at 4°C, the peptide was still intact. Upon incubation at 37°C, the initial peptide concentration was maintained for 7 h, but after 24 h the peptide was completely degraded. At room temperature, the peptide remained more or less stable for 16 h, after which a very fast degradation was observed and after 20 h incubation at 25°C the peptide could not be detected anymore.

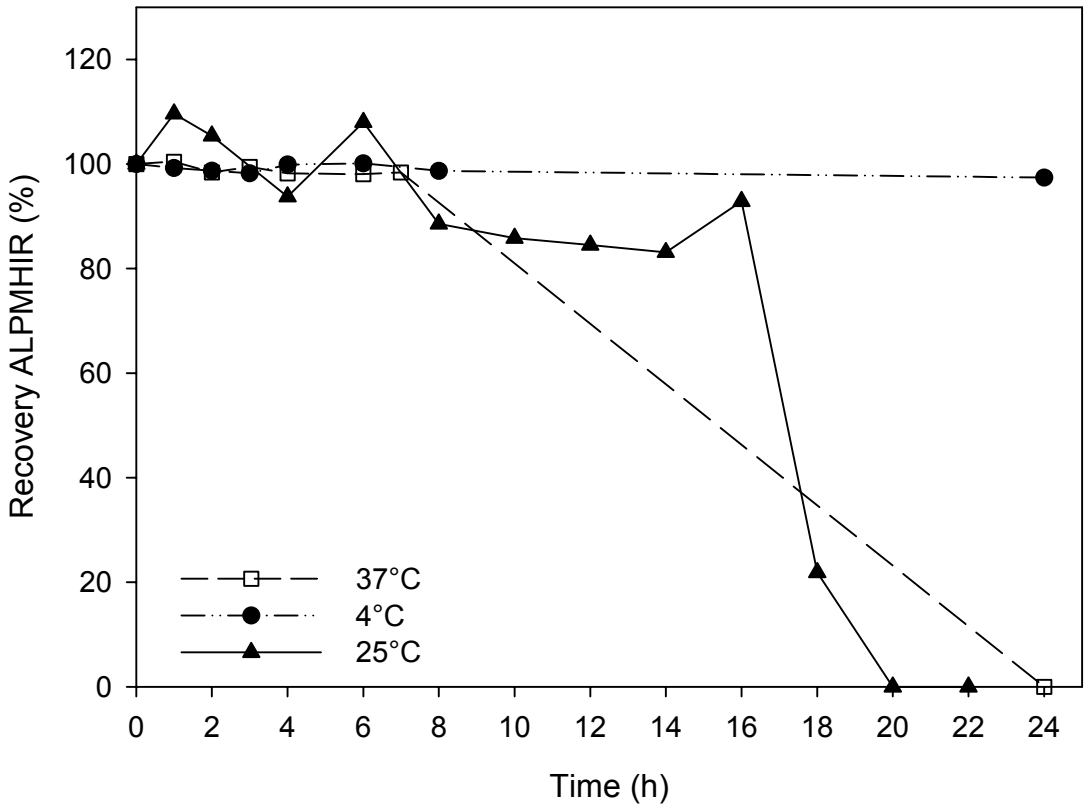

Figure 2. Recovery of the lactokinin Ala-Leu-Pro-Met-His-Ile-Arg in function of incubation time at 4°C, 25°C and 37°C, respectively.

In vitro gastrointestinal digestion

Theoretically, the lactokinin Ala-Leu-Pro-Met-His-Ile-Arg can be cleaved by both pepsin and α-chymotrypsin (Figure 3). Experimentally, the peptide was mainly degraded during the small intestine phase with trypsin and α-chymotrypsin hydrolysis at 37°C (Figure 4). However, at the end of the 4.5 h *in vitro* gastrointestinal digestion, 54 ± 4% of the lactokinin still remained intact. The major degradation products appeared to be two peptides that eluted

at 5.1-5.4 min and 12.2-12.4 min, respectively. The first peptide was identified by MALDI-TOF spectrometry as His-Ile-Arg: a peak with molecular mass 425 represented the tripeptide plus a proton, while 441 was the mass of the peptide plus proton in which methionine has been oxidised. The second peptide was not recovered (data not shown). Before digestion, 28.5-39.9% of the total peak area consisted of Ala-Leu-Pro-Met-His-Ile-Arg. His-Ile-Arg and the non-identified peak were only present for 0.6-1.0% and less than 0.3% respectively. After digestion, the lactokinin formed 13.6-32.0% of the total peak area; His-Ile-Arg and the other degradation product constituted respectively 5.8-7.0% and 4.0-5.6% of the total peak area. The control chromatograms of the pepsin solution and the pepsin-trypsin-α-chymotrypsin solution showed no peaks beyond a retention time of 4 min.

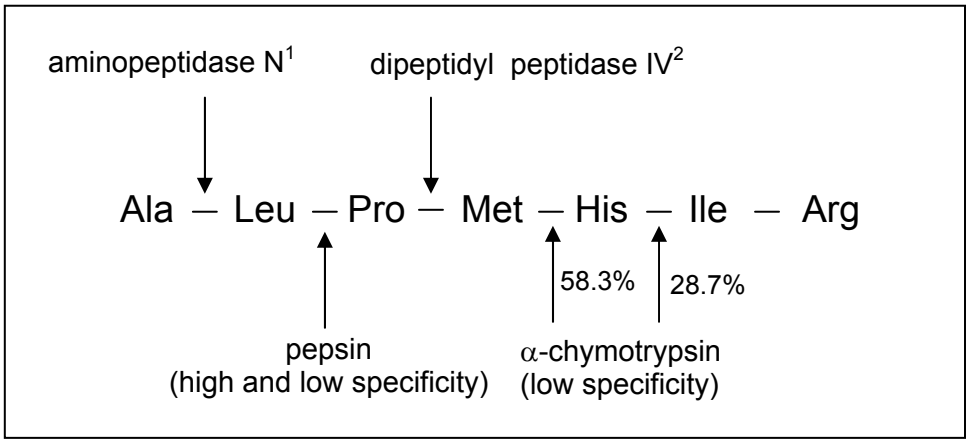

Figure 3. Possible cleavage sites (and probability of cleavage) of Ala-Leu-Pro-Met-His-Ile-Arg by gastrointestinal proteases (Peptide Cutter at www.expasy.ch) and brush border peptidases (Ganapathy and Leibach, 1999).

The ACE inhibitory activity of the peptide decreased over incubation time: after the stomach phase 92% of the initial ACE inhibitory activity was measured, while after the small intestine phase this value was reduced to 70% (Table 1).

Table 1. ACE inhibitory activity (%) during *in vitro* gastrointestinal digestion of Ala-Leu-Pro-Met-His-Ile-Arg: at the start (0 h), after the stomach phase (2 h) and after the small intestine phase (4.5 h) (n = 2).

Digestion time (h)	ACE inhibitory activity (%)
0	75 ± 2
2	69 ± 3
4.5	53 ± 8

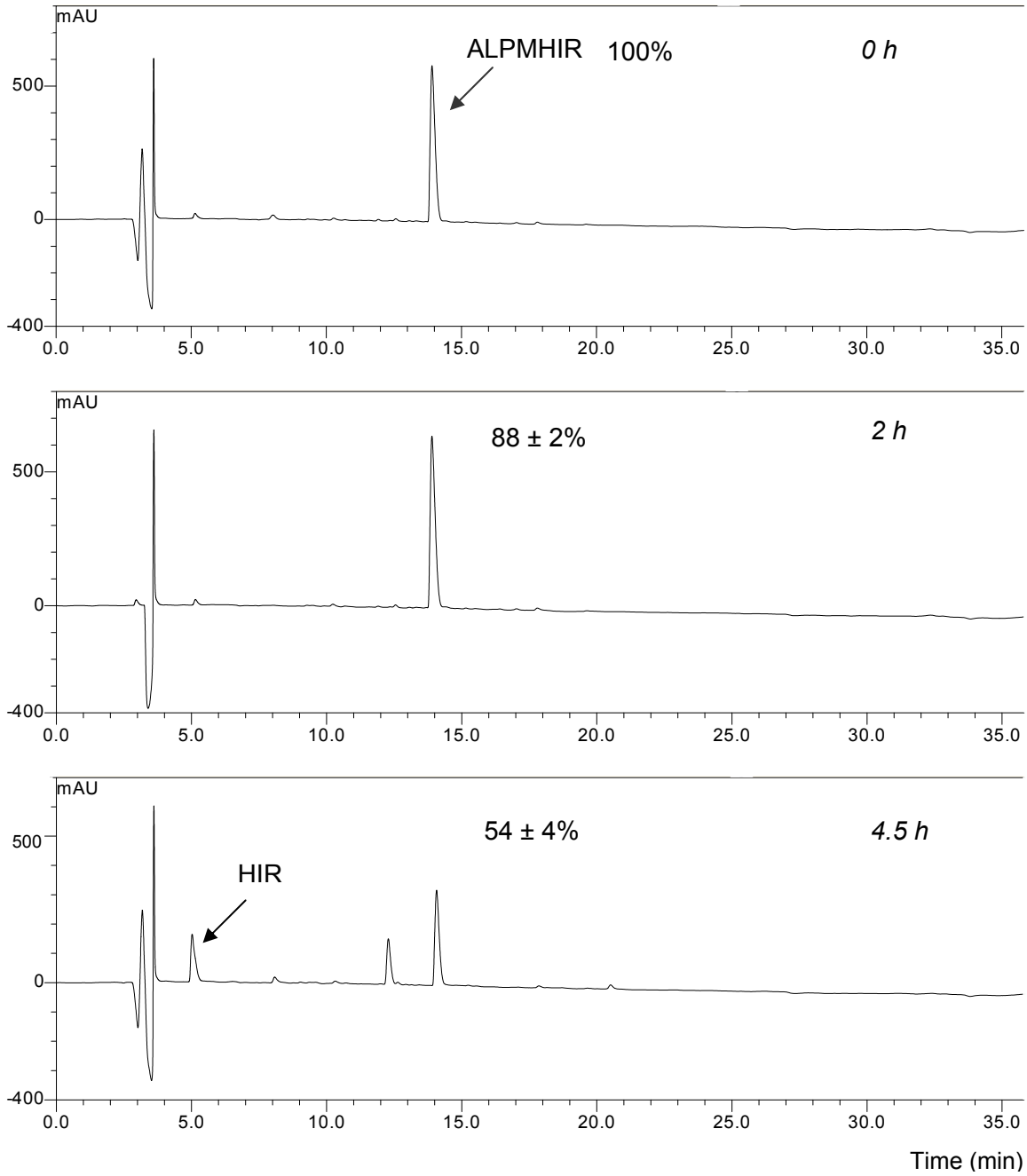

Figure 4. RP-HPLC chromatograms during *in vitro* gastrointestinal digestion of Ala-Leu-Pro-Met-His-Ile-Arg: at the start (0 h), after the stomach phase (2 h) and after the small intestine phase (4.5 h). The percent recovery of the peptide is mentioned (n = 2).

Stability towards peptidases from the intestinal brush border and enterocytes

A very rapid degradation of the lactokinin was observed in the presence of rat intestinal tissue peptidases. After 1 h incubation at 37°C in both the mucosal and serosal buffer solutions of an Ussing chamber experiment with rat intestinal tissue, the peptide was almost completely degraded. In the presence of Caco-2 cell homogenates for 3 h at 37°C, on the

contrary, no significant degradation of the lactokinin was observed (data not shown). In theory, brushborder peptidases like aminopeptidase N and subsequently dipeptidylpeptidase IV, which are also expressed in Caco-2 cells, are able to cleave the heptapeptide (Figure 3).

Transport experiments

Addition of 1 mM peptide to the mucosal compartment evoked a substantial increase in short-circuit current ΔI_{sc} of 0.4 $\pm$ 0.2 μA/cm^2. The transepithelial electrical resistance was about 300 Ω.cm^2 and remained constant throughout the experiment. Addition of 10 mM L-glutamine to the mucosal bathing solution at the end of the experiment induced an increase in short-circuit current ΔI_{sc} of 1.4 $\pm$ 0.9 μA/cm^2.

In the Ussing chamber without added peptide, neither ACE activity nor ACE inhibitory activity was observed in the mucosal or serosal compartment. The mucosal bathing solutions of all 6 Ussing chambers in which the ACE inhibitory peptide was supplemented, showed high ACE inhibitory activity (Figure 5). Only minor or no ACE inhibitory activity was detected in the serosal bathing solutions. However, when the samples were concentrated threefold, the ACE inhibitory activity in the serosal bathing solutions increased to 30%. Hereby, the effect of the increased chloride concentration in the threefold concentrated modified Krebs buffer on the ACE inhibitory activity was taken into account.

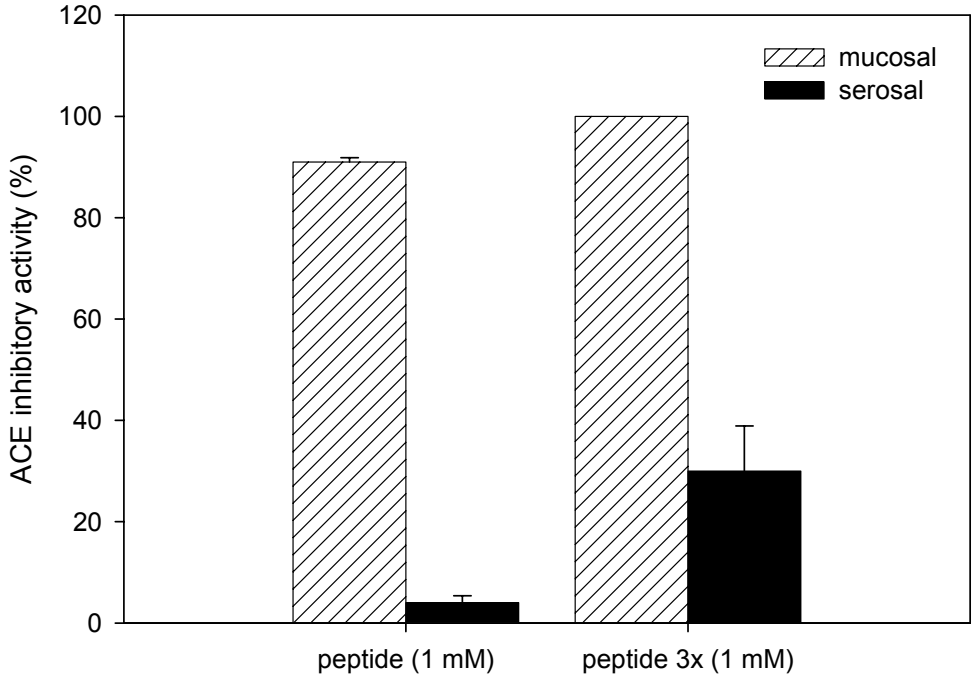

Figure 5. ACE inhibitory activity (%) observed in the mucosal and serosal compartments of the Ussing chambers with Caco-2 Bbe cell monolayer upon administration of the ACE inhibitory peptide Ala-Leu-Pro-Met-His-Ile-Arg (1 mM), analysed as such (n=6) and three times concentrated (n=2) in the ACE inhibition assay.

HPLC analysis of the mucosal samples yielded three significant peaks (not shown): peaks 1-2 with a retention time around 4 min and an area of 28% of the total peak area and peak 3 with a retention time of 29 min and an area of 59%. In the serosal samples only peaks 1-2 were detected after 4 min elution with a total area of 99%. Peaks 1-2 correspond to hydrophilic compounds present in the buffer solution like glucose and mannitol, while the last peak represents the heptapeptide. This peak closely resembled the one in the control HPLC chromatogram of the heptapeptide after synthesis (data not shown). MALDI-TOF spectrometry of both the mucosal and serosal samples revealed the presence of a peak at mass 837, which is the molecular mass of the heptapeptide (836) plus a proton (Figure 6). The mucosal sample contained a few other high molecular mass peaks: 859 is the mass of the heptapeptide plus sodium, while 881 represents the heptapeptide plus disodium. The peak with mass 853 in the serosal sample represents the peptide plus proton in which methionine has been oxidised.

Antihypertensive effect in SHR

During preliminary experiments, Ala-Leu-Pro-Met-His-Ile-Arg failed to reduce the blood pressure in SHR at a dose of 5 mg/kg BW.

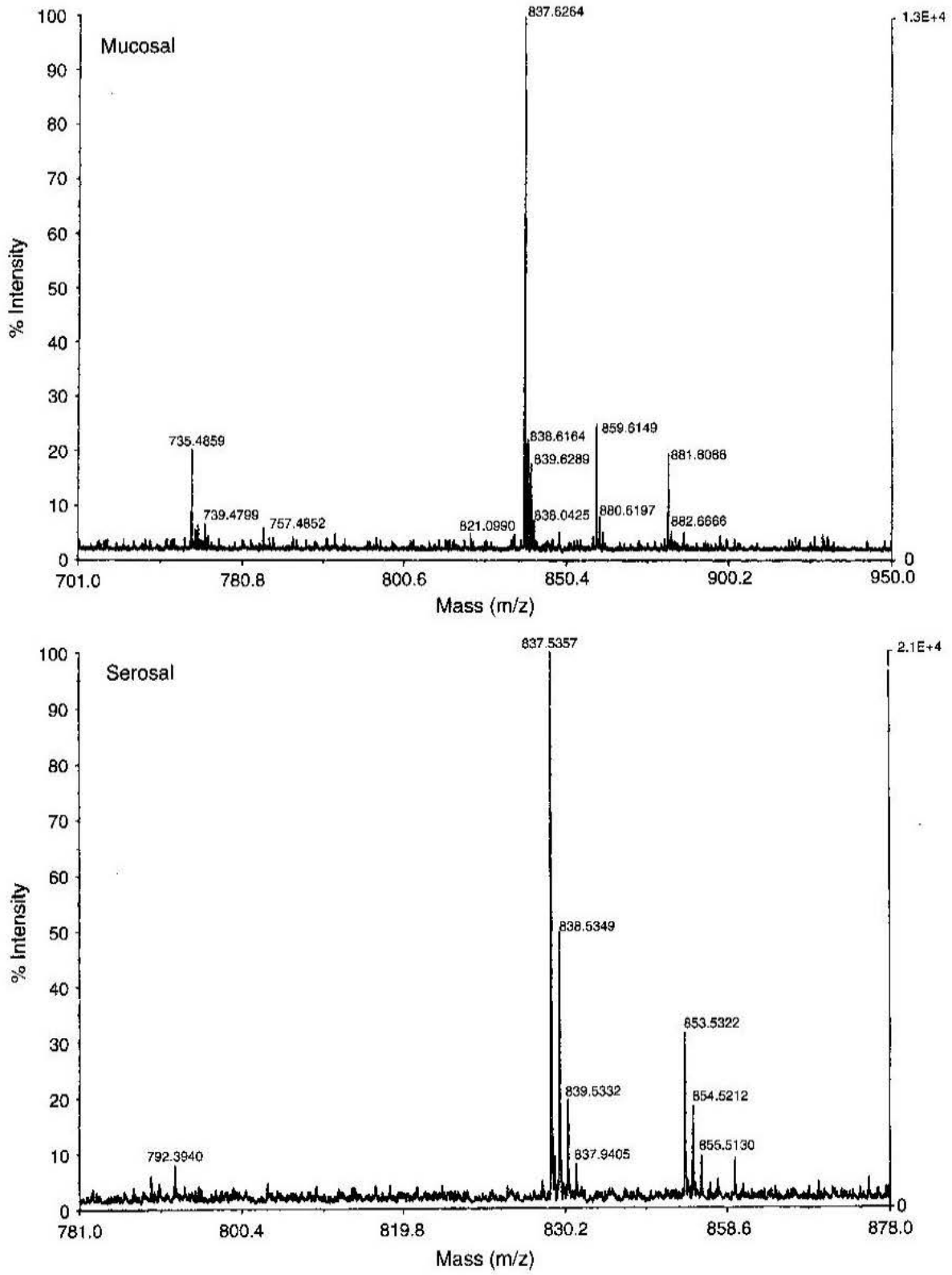

Figure 6. MALDI-TOF mass spectrometry profile of the mucosal and serosal compartment of the Ussing chamber upon administration of the ACE inhibitory peptide Ala-Leu-Pro-Met-His-Ile-Arg (1 mM).

DISCUSSION

Several *in vivo* studies have demonstrated the antihypertensive effect of orally administered food derived ACE inhibitory peptides (Hata *et al.*, 1996; Yoshii *et al.*, 2001). *In vitro* experiments may provide an outcome as laboratory-scaled alternatives for experimentation with humans or animals in the screening process for ACE inhibitory peptides. In this respect, the gastrointestinal digestion and intestinal transport of ACE inhibitory peptides are important facets in the *in vitro* investigation of the maintenance of ACE inhibitory activity through the oral pathway.

Stability towards chemical degradation and digestion

Ala-Leu-Pro-Met-His-Ile-Arg showed a rather good stability, which decreased of course with increasing storage temperature. Since the heptapeptide exhibited a thermal stability of 7 h at 37°C, and our digestion experiments at 37°C were shorter, the degradation observed during these experiments could be mainly attributed to the action of proteases.

Theoretically, the lactokinin, derived from a tryptic digest of β-lactoglobulin, can be cleaved by pepsin with high (pH lower than 2) and low specificity and by α-chymotrypsin with low specificity (Figure 3). The cleavage by pepsin is prevented in practice, however, by the unfavourable presence of proline. The same proline also impedes the action of α-chymotrypsin on the peptide bond between leucine and proline. His-Ile-Arg appears to be a major degradation product after α-chymotrypsin hydrolysis as the presence of methionine blocks the cleavage after histidine (Peptide Cutter at www.expasy.ch). At least half of the initial peptide concentration remained present after *in vitro* gastrointestinal digestion, which indicates a rather good stability of the peptide in the stomach and small intestine. Mullally *et al.* (1997b) also observed no hydrolysis of the peptide following incubation with pepsin and only a low degree of chymotryptic hydrolysis, which creates two extra peaks in the chromatogram at similar positions as in Figure 4. His-Ile-Arg and Ile-Arg display ACE inhibitory activity as well, although their IC_{50} values are rather high (954 µM and 696 µM) (Mullally *et al.*, 1996). We assume that the non-identified peptide is the longer and more hydrophobic fragment Ala-Leu-Pro-Met, which eluted at retention time 12.2-12.4 min. However, the degradation by brush border peptidases and the intestinal transport of these degradation products were not investigated; Ala-Leu-Pro-Met-His-Ile-Arg was the peptide under scrutiny. The ACE inhibitory activity was reduced to only 70% after the hydrolysis by stomach and pancreatic proteases, which can be due to the logistic relation between the ACE inhibitory activity and the inhibitor concentration, but also can indicate that the degradation

products contributed to the total ACE inhibitory activity. Consequently, the lactokinin resisted well the *in vitro* gastrointestinal digestion, although it is known that the luminal pancreatic proteases may be a metabolic obstacle in the oral delivery of even small peptides (Krondahl *et al.*, 2000).

The heptapeptide was very susceptible to hydrolysis by rat intestinal tissue peptidases, while in the presence of Caco-2 homogenates no significant breakdown was observed. Theoretically, aminopeptidase N, abundant in the intestinal brush border, can cleave alanine from the lactokinin, after which it can be further degraded by dipeptidyl peptidase IV, also a major brush border peptidase (Ganapathy and Leibach, 1999). These peptidases are normally also expressed in the Caco-2 cell monolayer, although it is known that certainly at the beginning of cell differentiation, the brush border membrane associated enzyme activities of Caco-2 cells are much lower than in the mammalian small intestine (Bolte *et al.*, 1998; Wilson *et al.*, 1990). Furthermore, species-specific differences in the enzymatic inactivation of biological active peptides in the intestine have been observed (Drucker *et al.*, 1997). On the other hand, it is suggested that the enzyme activity in the intestinal brush border of the rat is higher than in humans (Augustijns *et al.*, 1998). These facts, together with the lower concentration and longer storage time of the Caco-2 cell homogenates compared to the buffers containing rat peptidases, could explain the stability of the heptapeptide incubated in the presence of Caco-2 cell homogenates. Hence, compared to rat intestinal tissue, a less severe degradation of the lactokinin could be expected in the human intestine, increasing its potential to reach the cardiovascular system intact.

Intestinal transport

In this *in vitro* study, the mass spectrometry results combined with the electrophysiological and ACE inhibition data show that transport of intact Ala-Leu-Pro-Met-His-Ile-Arg through the Caco-2 Bbe monolayer took place within 10 min after administration.

The small increase in short-circuit current ΔI_{sc} after addition of the peptide to the mucosal compartment reflects the transport of the positively charged heptapeptide (Arg), transport of its charged degradation products or ion transport induced by the heptapeptide. As was the case in our study, addition of L-glutamine to the mucosal compartment leads in a viable and functional small intestinal cell monolayer to an increase in short-circuit current as a result of the cotransport of sodium. The transepithelial electrical resistance of 300 $\Omega.cm^2$ reflects the structural integrity of the cell monolayer (Wilson *et al.*, 1990).

The Caco-2 Bbe cell monolayers did not secrete ACE enzyme or ACE inhibitory compounds. The ACE inhibition assay indicated a high ACE inhibitory activity in the mucosal

compartments of the Ussing chambers. This could be due to the heptapeptide or to active degradation products. Substantial ACE inhibitory activity was only detected in the serosal compartments of the Ussing chambers after concentrating the samples.

HPLC analysis clearly detected the pure peptide in the mucosal compartments. The peak of the heptapeptide was not retrieved in the analysis of the serosal compartments. However, the highly sensitive MALDI mass spectrometry demonstrated the presence of the heptapeptide in both the mucosal and serosal samples.

Although it cannot be excluded that some degradation of the heptapeptide occurred or that transport of degradation products took place, these results show that the ACE inhibitory heptapeptide is transported intact, be it at very low concentrations. As samples were taken 10 min after addition of the ACE inhibitory peptide to the mucosal compartment, there may have been insufficient time for the transport to occur completely. However, longer sampling times could increase the risk of degradation. Few studies describe the *in vitro* transepithelial transport of bioactive oligopeptides. They show that the susceptibility to brushborder peptidases controls the transport rate, which is in agreement with our findings, and that several transport mechanisms may participate in oligopeptide passage across the intestinal epithelium (Matsui *et al.*, 2002a; Shimizu *et al.*, 1997). Another study in the Caco-2 cell model illustrates that, although the ACE inhibitory peptide Val-Pro-Pro is detected in the aorta after oral administration and exerts an antihypertensive effect, less than 2% of the peptide concentration added to the apical chamber is transported to the basolateral side in 60 min (Satake *et al.*, 2002). Hence, despite the low transport of the lactokinin observed *in vitro*, it may reach the systemic circulation *in vivo* in substantial amounts to exert a biological activity.

Antihypertensive activity

In some preliminary experiments, the lactokinin did not produce antihypertensive activity at a dose of 5 mg/kg BW upon intravenous injection in SHR. Either the peptide is rapidly degraded by plasma peptidases or just inactive *in vivo* at this dose, which both indicate that aministration of a higher dose may lead to a reduction in blood pressure. Further research needs to elucidate this.

In conclusion, Ala-Leu-Pro-Met-His-Ile-Arg resisted *in vitro* gastrointestinal digestion well, but is susceptible to degradation by peptidases from the rat intestinal brush border and enterocytes. Furthermore, it is transported intact trough a Caco-2 cell monolayer, although at a very low concentration.

ACKNOWLEDGEMENTS

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CHAPTER 4

ACE INHIBITORY FERMENTS AND *IN VITRO* DIGESTS FROM PEA AND WHEY PROTEIN

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Chapter 4

ACE inhibitory ferments and in vitro digests of pea and whey protein

ABSTRACT

Pea and whey protein were fermented by *Lactobacillus helveticus* and *Saccharomyces cerevisiae* in monoculture and in combination at 28 and 37°C in order to release Angiotensin I Converting Enzyme (ACE) inhibitory peptides. The fermentation products were subjected to *in vitro* gastrointestinal digestion and the digests of non-fermented protein solutions served as controls. After fermentation, the ACE inhibitory activity (%) of 2.73 mg/ml samples increased by 18 to 30% for all treatments, except for the fermentations of whey protein by *Saccharomyces cerevisiae* at 28°C, where no significant change was observed. After digestion, however, both fermented and non-fermented samples at a concentration of 2.73 mg/ml reached maximal ACE inhibitory activity (%). The whey digests tended to have lower 50% inhibitory concentrations (IC_{50}) (0.148 to 0.072 mg/ml), hence higher ACE inhibitory activity, than the pea digests (0.183 to 0.093 mg/ml). The non-fermented samples were at least as ACE inhibitory active as the fermented ones. The non-fermented whey protein digest showed the highest ACE inhibitory activity of all. For pea protein, the non-fermented sample had the one but lowest IC_{50} value. These results suggest that *in vitro* gastrointestinal digestion was the predominant factor controlling the formation of ACE inhibitory activity, hence indicating its importance in the bioavailability of ACE inhibitory peptides.

Key words: ACE inhibitory peptides, fermentation, gastrointestinal digestion, *Lactobacillus helveticus*, *Saccharomyces cerevisiae*

INTRODUCTION

Hypertension is a major risk factor for the development of cardiovascular disease, which is one of the main causes of mortality in Western countries (Duprez *et al.*, 2002). Diet and lifestyle modification represent effective tools in the prevention of hypertension. In the treatment of the disease, these diet and lifestyle changes can decrease requirements of antihypertensive medication, as well as have beneficial effects on the metabolic aberrations related to hypertension not remedied by most drugs (Hermansen, 2000). In this respect, functional foods with blood pressure lowering properties have recently received considerable attention.

Angiotensin I converting enzyme (ACE; EC 3.4.15.1) is a dipeptidyl carboxypeptidase that elevates blood pressure by producing the vasoconstrictor angiotensin II and degrading the vasodilator bradykinin (Campbell, 1987). ACE inhibitory peptides have already been isolated from many food proteins (Dziuba *et al.*, 1999a; Fitzgerald and Meisel, 2000). Fermentation and/or digestion are popular food processing steps to release these bioactive or functional peptides from food proteins (Abubakar *et al.*, 1998; Gobbetti *et al.*, 2000). After oral administration, the ACE inhibitory peptides may exert an antihypertensive effect, provided they pass the gastrointestinal digestive and absorptive system and reach the cardiovascular system in an active form.

Recently, certain functional foods containing ACE inhibitory peptides have been shown to act as an additional or alternative treatment in hypertension. Daily administration of Calpis sour milk to hypertensive human subjects significantly reduces their blood pressure. The antihypertensive effect of this milk fermented by a starter containing *Lactobacillus helveticus* and *Saccharomyces cerevisiae*, is due to the presence of the ACE inhibitory peptides Val-Pro-Pro and Ile-Pro-Pro, contained in the primary structure of β-casein, and β-casein and κ-casein, respectively (Takano, 1998). Moreover, long-term intake of Val-Pro-Pro and Ile-Pro-Pro, or a sour milk containing these tripeptides attenuates the development of hypertension in spontaneously hypertensive rats (SHR), suggesting a preventive role of ACE inhibitory peptides in hypertension as well (Sipola *et al.*, 2001).

Pea protein is a valuable protein for human nutrition with the advantage of a good balanced profile of amino acids, a high content in the essential amino acid lysine (82 g/kg protein) and a rather good solubility. In addition, it is an environmentally friendly, low input crop and may represent an alternative to soy. ACE inhibitory peptides derived from pea protein have not yet been reported in literature. In a previous study high ACE inhibitory activity in a tryptic digest of pea protein isolate was found, suggesting that pea may be an

alternative source of ACE inhibitory peptides (Vermeirssen *et al.*, 2002b). Fermentation was investigated as a means to produce ACE inhibitory peptides and the ACE inhibitory activity derived from pea protein was compared to the one of whey protein, a known source of ACE inhibitory peptides (Pihlanto-Leppälä, 2001). Eight lactobacilli and *Saccharomyces cerevisiae*, microorganisms already used in food processing and consequently generally recognised as safe (GRAS), were used to inoculate the protein media. The combination of yeast and lactic acid bacteria is often used in fermented milk based products as several synergistic interactions may occur (Viljoen, 2001). The importance of the gastrointestinal proteases pepsin (A, EC 3.4.23.1), trypsin (EC 3.4.21.4) and α-chymotrypsin (EC 3.4.21.1) on the formation and/or degradation of ACE inhibitory peptides was also assessed.

MATERIALS AND METHODS

Products

The pea protein isolate Pisane® HD and the (rennet) whey protein isolate Lacprodan® DI-9213 were obtained from Cosucra SA (Fontenoy, Belgium) and Acatris Belgium NV (Londerzeel, Belgium), respectively. Thiamin, pyridoxine, D-pantothenic acid, inositol, D-biotin, nicotinic acid, riboflavin, pepsin (P 6887), trypsin (T 1426), α-chymotrypsin (C 4129), trichloroacetic acid solution (490-10), ACE reagent (305-10), ACE control-E (A 7040), 100% (w/v) trifluoroacetic acid solution (30,203-1) were purchased from Sigma-Aldrich (St.-Louis, MO, USA). Non-specified products were analytical grade from VWR International (Zaventem, Belgium)

Fermentation

The following lactobacilli were used in the screening fermentations: *Lactobacillus fermentum* LMG8900 (intestine from 8 day old breastfed baby), *Lactobacillus gasseri* LMG9203 (human), *Lactobacillus oris* LMG9848 (human Italian saliva), *Lactobacillus reuteri* LMG9213 (human intestine), *Lactobacillus acidophilus* LMG7943 (human), *Lactobacillus plantarum* LMG9211 (human saliva), *Lactobacillus plantarum* LMG9212 (human saliva), *Lactobacillus helveticus* LMG 11474 (Swiss cheese starter) (LMG Culture Collection, Ghent University, Belgium). In the subsequent fermentations, *Saccharomyces cerevisiae* (commercial baking yeast) was also used. Lactobacilli were propagated in MRS broth (de Man, Rogasa, Sharpe) (Oxoid, Basingstoke, UK) under micro-aerophilic conditions at 37°C

for 24 h and baking yeast in YPD broth (10 g/l Yeast extract (Oxoid), 10 g/l Peptone bacteriological (Oxoid), 20 g/l D-glucose) under aerobic conditions at 28°C for 24 h.

The screening fermentations were carried out with the lactobacilli described above on pea protein only, for 48 h at 37°C. Fermentations with *L. helveticus* and *S. cerevisiae* occurred in monoculture at their optimal temperature (37 and 28°C respectively) and in combination (37 and 28°C) for 48 h on both pea and whey protein. At the start of the fermentation the selected microorganisms were added to the fermentation medium under sterile conditions in a concentration of 6.3 $\log_{10}$ CFU/ml for *L. helveticus* and 5.7 $\log_{10}$ CFU/ml for *S. cerevisiae*. The inoculated fermentation medium was incubated at 28 or 37°C for 48 h. Fermentation experiments with *L. helveticus* or/and *S. cerevisiae* were repeated at least three times. Samples were taken before and after fermentation and after *in vitro* gastrointestinal digestion.

The fermentation medium was composed as follows: 40 g/l (4 % (w/v)) protein, 20 g/l D-glucose, 10 ml/l vitamin solution, 10 ml/l salt solution, 0.1 M sodium phosphate buffer, pH 6 to 6.5. To avoid the Maillard reaction, the protein solution and the rest solution, consisting of D-glucose and salt solution in sodium phosphate buffer, were autoclaved separately for 15 min at 121°C, 101.3 kPa overpressure. The salt solution contained 5 g/l $MgSO_4.7H_2O$, 5 g/l KH_2PO_4, 1 g/l $CaCl_2.2H_2O$ and 0.5 g/l $MnSO_4.H_2O$. The vitamin solution consisted of 40 mg/l thiamine, 40 mg/l pyridoxine, 40 mg/l D-pantothenic acid, 200 mg/l inositol, 2 mg/l D-biotin, 40 mg/l nicotinic acid, 40 mg/l riboflavin. It was added to the autoclaved fermentation medium after sterilisation by 0.22 µm filtration (Millipore, Bedford, MA, USA). Prior to inoculation the pH of the 100 ml whey protein fermentation medium was adjusted to pH 6 to 6.5 by addition of 450 µl 10 M NaOH.

Digestion

Fermented and non-fermented protein solutions were subsequently digested. The conditions of the physiological digestion were based on literature (De Boever *et al.*, 2000; Ganong, 1997; Gauthier *et al.*, 1986). To simulate the digestion in the stomach, the pH of the fermented medium was adjusted to 2 with 10 M and 1 M HCl under rigorous mixing. Subsequently, pepsin was supplemented in an E/S of 1/250 (w/w) and the medium was incubated on a shaker for 2 h at 37°C. Next, the small intestinal digestion was simulated by setting the pH at 6.5 with 10 M and 1 M NaOH under rigorous mixing and by addition of trypsin and α-chymotrypsin, both in an E/S of 1/250 (w/w), followed by another incubation for 2.5 h on a shaker at 37°C. At the end of digestion the pH was adjusted to 5 with 10 M and

1 M HCl. As this is near the iso-electrical point for both proteins (pea: pH 4.5, whey: pH 4 to 5), a standardised and clear separation was obtained by subsequent centrifugation.

Follow up of the fermentation

At the start and the end of the fermentation, pH was measured using a 744 pH Meter (Metrohm, Herisau, Switzerland) and plate counts were performed after 72 h incubation at 37°C on Rogosa agar (Oxoid) and at 28°C on YPD agar (YPD broth + 20 g/l agar) for lactobacilli and *S. cerevisiae*, respectively.

Degree of proteolysis

The degree of proteolysis was determined by the ratio of the non-protein Kjeldahl nitrogen to the total Kjeldahl nitrogen. Samples for non-protein nitrogen determination were treated with trichloroacetic acid solution to a final concentration of 6% (w/v), shaken for 5 min and then centrifuged at 12 000 g for 10 min at 4°C. This supernatant and a sample for total nitrogen determination were stored at -80°C prior to analysis.

ACE inhibitory activity

Samples for ACE inhibition were centrifuged at 10 000 g for 15 min at 4°C, the supernatant was frozen in liquid nitrogen and stored at -80°C. Freezing in liquid nitrogen was preferred over heating at 98°C for 10 min to inactivate the proteases and to maintain the bioactivity of the peptides. Next, the frozen samples were lyophilised to obtain a dry powder, which was analysed by the ACE inhibition assay 3 as described in Chapter 2.

Protease activity

The protease activity was determined on the supernatant and the redissolved precipitate of the 24 h propagated culture of *L. helveticus* and *S. cerevisiae* respectively, after centrifugation at 5000 g for 10 min (n = 3). Activity was assessed by means of a commercial available Universal Protease Substrate spectrophotometric assay (Roche Diagnostics, Basel, Switzerland).

HPLC

Samples for the HPLC analysis were treated as for the ACE inhibition assay. Twenty milligram lyophilised powder was dissolved in 2 ml milli-Q water (Millipore) and ultrafiltrated-centrifuged in Centricon YM-3000 tubes (MWCO = 3000 Da) (Millipore) for 2 h at 7500 g.

The permeate was analysed by reversed-phase HPLC on a Prosphere 300 Å C_{18} column (250 x 4.6 mm, 5 µm) (Alltech Associates, Deerfield, IL, USA) and a Dionex (Sunnyvale, CA, USA) HPLC with an autosampler ASI-100, pump series P580, STH585 column oven, UV-VIS detector UVD340S operating at 210 nm and Chromeleon 6.0 software. The temperature was controlled at 25°C and the flow rate was maintained at 1 ml/min. The permeates were eluted by a linear gradient from 90% solvent A (H_2O + 0.1% (w/v) TFA) to 50% solvent B (acetonitrile + 0.085% (w/v) TFA) in 30 min, again to 90% solvent A in the next 20 min and remaining at 90% solvent A the last 10 min.

Sonication

Sonication (Sonicator 250, Branson, G. Heinemann, Schwäbisch Gmünd, Germany) was performed in the ice-cooled 24 h propagated culture of *L. helveticus*, *S. cerevisiae* or a combination of both (1/1 (v/v) propagated culture of both microorganisms) to release the intracellular proteases from the lysed cells in the medium. Operational parameters resulting in the highest protease activity as obtained with the commercial protease assay were found to be: time 12 min, output control 3 and duty cycle 70%. A 4% (w/v) whey or pea protein solution in demineralised water was incubated with 1% (v/v) lysed cell suspensions of *S. cerevisiae* at 28°C or *L. helveticus* or both at 37°C for 24 h.

Statistical analysis

All values are reported as mean ± standard error (SE) of the mean (n_{min} = 3). Some missing values (2% of the dataset) were replaced by the average of the treatments. A (paired) Student t-test studied the significance of changes in pH, lactobacilli and yeast counts during fermentation. To exclude the initial effect of the protein itself, the statistical analysis was performed on the changes in degree of proteolysis and ACE inhibitory activity after fermentation, and after fermentation and digestion. By means of the General Linear Model procedure (Minitab 11.21, State College, PA, USA) significant differences in type of protein (p_{prot}) and type of fermentation (p_{ferm}) were assigned. When there was a significant type of protein × type of fermentation interaction, for both proteins a Oneway ANOVA analysis was carried out in type of fermentation. All data used in the variance analysis met the homogeneity of variance requirement assessed by Bartlett's test for normal and Levene's test for not-normal distributions. Normality was tested by means of the Anderson-Darling criterion. Indication of subgroups was done by Tukey post hoc test ($p < 0.05$). The statistical analysis was performed on the log IC_{50} value, as this value is calculated from the logistic model and is normally distributed.

RESULTS

Screening of lactobacilli

Eight different GRAS lactobacilli were screened for the production of ACE inhibitory activity during fermentation and subsequent *in vitro* gastrointestinal digestion of pea protein (Table 1). Fermentation by *L. helveticus* yielded the highest ACE inhibitory activity and was therefore selected for subsequent experimentation. After *in vitro* digestion all ferments reached high to maximal ACE inhibitory activity.

Table 1. ACE inhibitory activity (%) after fermentation of pea protein medium by different lactobacilli at 37°C and subsequent *in vitro* digestion. The data represent the result of a single experiment.

Strain	After fermentation[1,2]	After *in vitro* digestion[1,3]
Lactobacillus fermentum LMG8900	12	90
Lactobacillus gasseri LMG9203	12	96
Lactobacillus oris LMG9848	16	100
Lactobacillus reuteri LMG9213	9	96
Lactobacillus acidophilus LMG7943	13	93
Lactobacillus plantarum LMG9211	7	83
Lactobacillus plantarum LMG9212	11	99
Lactobacillus helveticus LMG11474	37	96

[1] The concentration of ferment or digest in the assay was 2.73 mg/ml.
[2] The initial ACE inhibitory activity of the pea fermentation medium was 16 ± 2%.
[3] The ACE inhibitory activity of non-fermented digested pea fermentation medium amounted to 99.3 ± 0.7%.

Fermentation by *Lactobacillus helveticus* or/and *Saccharomyces cerevisiae*

Autoclaved pea and whey protein medium were fermented by *L. helveticus* at 37°C, *S. cerevisiae* at 28°C or a combination of both microorganisms at 28 or 37°C in order to produce ACE inhibitory active ferments. Fermentation was always followed by an *in vitro* gastrointestinal digestion with pepsin at pH 2 and a combination of trypsin and α-chymotrypsin at pH 6.5. Non-fermented autoclaved pea and whey protein medium, subjected to *in vitro* gastrointestinal digestion, served as control experiments.

The presence of *L. helveticus* at its optimal temperature of 37°C initiated a large pH decrease during fermentation ($p_{ferm} < 0.001$) (Table 2). The pH after fermentation was lower

for pea than for whey (p_{prot} < 0.001). *L. helveticus*' counts increased during fermentation in monoculture on both protein media. However, this lactic acid bacterium hardly grew in the presence of the yeast, both at 37 and 28°C, for pea and whey protein (Table 2). *S. cerevisiae*'s counts increased during fermentation at 28°C, whether or not in the presence of *L. helveticus*. A higher, non-optimal temperature, e.g. 37°C, was disadvantageous for the yeast. Hence, the growth of both microorganisms was affected by the type of fermentation (p_{ferm} < 0.001), but in a different manner. The counts of both microorganisms did not differ significantly between the two proteins (*L. helveticus*: p_{prot} = 0.209; *S. cerevisiae*: p_{prot} = 0.938).

Table 2. pH and plate counts (log_{10} CFU/ml) of *Lactobacillus helveticus* and *Saccharomyces cerevisiae* at the start and after 48 h fermentation for the different types of fermentations (LH37: *L. helveticus* at 37°C, Y28: *S. cerevisiae* at 28°C, LHY37: *L. helveticus* and *S. cerevisiae* at 37°C, LHY28: *L. helveticus* and *S. cerevisiae* at 28°C), for both pea and whey protein (n_{min} = 3).

		pH	*L. helveticus*	*S. cerevisiae*
Pea	Start	6.1 ± 0.0	6.3 ± 0.1	5.7 ± 0.1
	LH37	3.5 ± 0.1***[a]	7.8 ± 0.4**[a]	/
	Y28	5.5 ± 0.2[b]	/	7.1 ± 0.2**[a]
	LHY37	3.6 ± 0.1**[a]	6.5 ± 0.3[b]	5.6 ± 0.5[b]
	LHY28	5.4 ± 0.3[b]	6.6 ± 0.4[b]	7.7 ± 0.2*[a]
Whey	Start	6.3 ± 0.0	6.3 ± 0.1	5.7 ± 0.1
	LH37	3.9 ± 0.1***[c]	7.4 ± 0.3**[a]	/
	Y28	6.1 ± 0.0**[d]	/	7.6 ± 0.1**[a]
	LHY37	4.3 ± 0.2***[c]	6.3 ± 0.3[b]	5.1 ± 0.2**[b]
	LHY28	5.9 ± 0.1*[d]	6.3 ± 0.2[b]	7.6 ± 0.3***[a]

/ = not applicable.
Significant from the start value: *p < 0.05, **p < 0.01, ***p < 0.001.
Different letters indicate significant differences in 'type of fermentation' (and 'type of protein') (p < 0.05).

Degree of proteolysis and ACE inhibitory activity in ferments and digests

At the start of the fermentation process the degree of proteolysis for pea, 6.7 ± 0.4%, was lower than for whey, 17.9 ± 0.5% (p < 0.001). The ACE inhibitory activity of the fermentation medium of pea amounted to 16 ± 2%, while for whey this was 63 ± 2% (p < 0.001). Figure 1 shows the degree of proteolysis and Figure 2 the ACE inhibitory activity, after fermentation and after fermentation and digestion for both proteins. The statistical analysis, however, was

performed on the changes of these parameters during fermentation, and fermentation and digestion, in order to exclude the initial degree of proteolysis and ACE inhibitory activity of the protein itself.

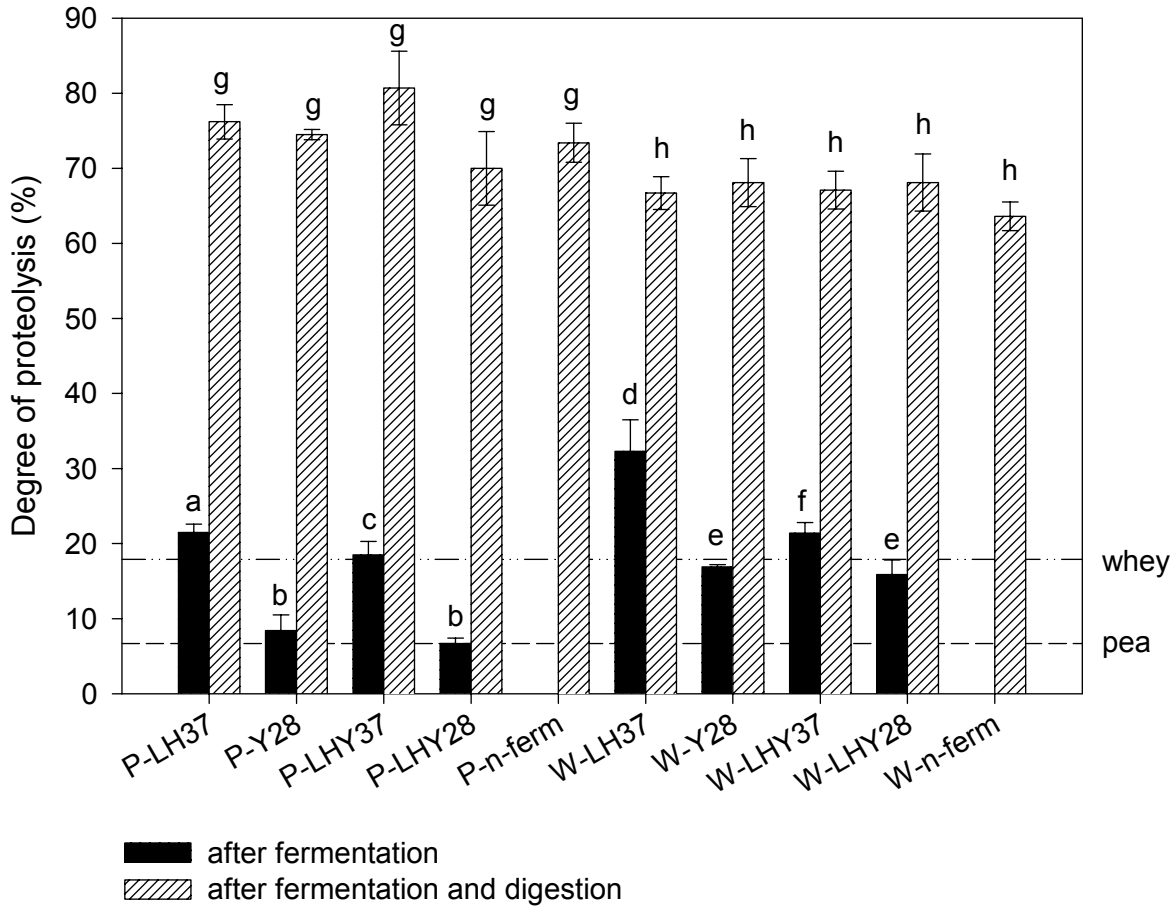

Figure 1. Degree of proteolysis (%) after fermentation, and after fermentation and digestion of pea (P) and whey (W) protein (n_{min} = 3). (LH37: *Lactobacillus helveticus* at 37°C, Y28: *Saccharomyces cerevisiae* at 28°C, LHY37: *L. helveticus* and *S. cerevisiae* at 37°C, LHY28: *L. helveticus* and *S. cerevisiae* at 28°C, n-ferm: non-fermented). Straight, dashed lines indicate the initial degree of proteolysis of the pea and whey medium. Different letters indicate significant differences in the change ($p < 0.05$).

The change in degree of proteolysis during fermentation was significantly different between the two proteins (p_{prot} = 0.033) and between the different types of fermentation ($p_{ferm} < 0.001$). The degree of proteolysis increased during fermentation maximally by 15%, namely in the monoculture fermentation by *L. helveticus* (Figure 1). The fermentation by both *L. helveticus* and *S. cerevisiae* at 37°C also augmented the degree of proteolysis, and more in the pea than in the whey protein medium. In the presence of *S. cerevisiae* at 28°C, no

significant change could be observed, except for the monoculture fermentation by *S. cerevisiae* on whey protein (W-Y28), where the degree of proteolysis slightly decreased after fermentation ($p < 0.05$). After *in vitro* digestion, the degree of proteolysis augmented sharply in all samples to 75 ± 2% for pea and 67 ± 1% for whey and the degree of proteolysis in the non-fermented media did not differ from the fermented ones ($p_{ferm} = 0.625$). The less pronounced increase in degree of proteolysis for the whey protein during digestion, compared to the pea protein ($p_{prot} < 0.001$), may partially be explained by the higher initial degree of proteolysis for the whey protein fermentation medium.

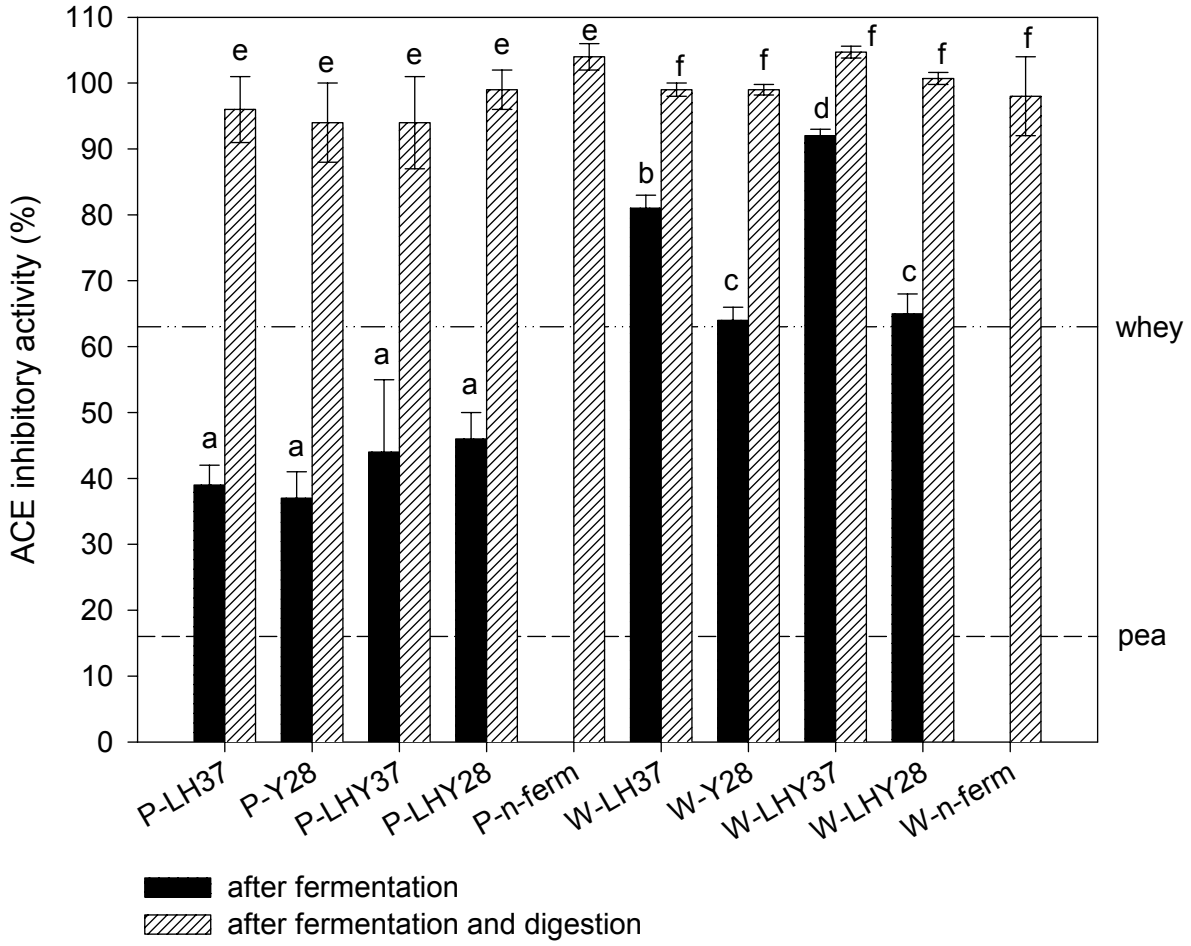

Figure 2. ACE inhibitory activity (%) after fermentation, and after fermentation and digestion of pea (P) and whey (W) protein ($n_{min} = 3$). (LH37: *Lactobacillus helveticus* at 37°C, Y28: *Saccharomyces cerevisiae* at 28°C, LHY37: *L. helveticus* and *S. cerevisiae* at 37°C, LHY28: *L. helveticus* and *S. cerevisiae* at 28°C, n-ferm: non-fermented). Straight, dashed lines indicate the initial ACE inhibitory activity of the pea and whey medium. Different letters indicate significant differences in the change ($p < 0.05$).

For the pea protein, the type of fermentation did not influence the increase in percent ACE inhibitory activity of a 2.73 mg/ml sample after fermentation (p_{ferm} = 0.539) (Figure 2). For the whey protein, on the other hand, the presence of *L. helveticus* and a temperature of 37°C resulted in increased ACE inhibitory activity after fermentation, whereas the presence of *S. cerevisiae* and a temperature of 28°C did not (p_{ferm} < 0.001) ($p_{interaction}$ = 0.008). *In vitro* digestion augmented the ACE inhibitory activity significantly and both non-fermented and fermented samples reached the maximal level of 100% (p_{ferm} = 0.959). Somewhat due to the high initial ACE inhibitory activity of the whey protein, the increase during digestion was smaller compared to the pea protein (p_{prot} < 0.001). The degree of proteolysis did not correlate with the ACE inhibitory activity: although the pea ferments at 28°C showed a lower degree of proteolysis compared to the pea ferments at 37°C, the ACE inhibitory activity of both groups did not differ. The IC_{50} value enabled to further distinguish the ACE inhibitory activity of the digests (Table 3).

Table 3. IC_{50} (mg/ml) and their log values on which the statistical analysis was performed, for the different types of fermentation for pea and whey protein after *in vitro* digestion (n_{min} = 3). (LH37: *Lactobacillus helveticus* at 37°C, Y28: *Saccharomyces cerevisiae* at 28°C, LHY37: *L. helveticus* and *S. cerevisiae* at 37°C, LHY28: *L. helveticus* and *S. cerevisiae* at 28°C, n-ferm: non-fermented).

		IC_{50}	Log IC_{50} ± SE
Pea	LH37	0.183	-0.74 ± 0.02^{a}
	Y28	0.158	-0.80 ± 0.02^{a}
	LHY37	0.162	-0.79 ± 0.00^{a}
	LHY28	0.093	-1.03 ± 0.07^{b}
	n-ferm	0.121	-0.92 ± 0.08^{ab}
Whey	LH37	0.148	-0.83 ± 0.02^{c}
	Y28	0.078	-1.11 ± 0.01^{de}
	LHY37	0.109	-0.96 ± 0.03^{cd}
	LHY28	0.094	-1.03 ± 0.02^{de}
	n-ferm	0.072	-1.14 ± 0.07^{e}

Different letters indicate significant differences (p < 0.05).

The type of fermentation influenced the (log) IC_{50} values after *in vitro* digestion for both proteins in a dissimilar way ($p_{interaction}$ = 0.012). The pea digest with the combined fermentation at 28°C showed the lowest IC_{50}, although this did not differ significantly from the

non-fermented pea digest (p_{ferm} = 0.018). With the exception of this fermented pea digest, the whey digests tended to have a lower IC_{50}, hence a higher ACE inhibitory activity, than the corresponding pea digests. The presence of the yeast at 28°C seemed to decrease the IC_{50} value. The lowest IC_{50} value was obtained in the non-fermented whey protein medium, although this value was not significantly different from whey digests fermented by the yeast at 28°C (p_{ferm} = 0.004). Hence, the non-fermented samples were at least as ACE inhibitory active as the fermented ones.

Protease activity

Protease activity in the 24 h propagated culture of *L. helveticus* and *S. cerevisiae* was measured on the supernatant and the cell precipitate in order to identify the origin of protease activity. The protease activity in the supernatant measured 0.80 ± 0.01 U/L for *L. helveticus* and 0.53 ± 0.07 U/L for *S. cerevisiae*. The cell precipitate of *L. helveticus* had a protease activity of 0.39 ± 0.03 U/L, whereas the one of *S. cerevisiae* had an activity of 0.55 ± 0.13 U/L.

HPLC profiles

The HPLC profile of the samples provided a fingerprint of the small peptides (MWCO ultrafiltration-centrifugation = 3000 Da) present in the digests. No striking differences between the chromatograms of the fermented and the non-fermented digests were observed. For both proteins, the HPLC chromatogram of one fermented digest and the non-fermented digested medium are displayed in Figure 3.

Contribution of intracellular proteases

Non-autoclaved 4% (w/v) pea and whey protein solution had a degree of proteolysis of respectively 2.4 ± 0.4% and 12.9 ± 0.7% ($p < 0.001$), and an ACE inhibitory activity of respectively 10.0 ± 0.7% and 15 ± 2% ($p = 0.083$). After 24 h incubation with a suspension of lysed cells of *L. helveticus* at 37°C, *S. cerevisiae* at 28°C and a combination of both at 37°C, the degree of proteolysis only augmented for the pea protein and amounted to 30% at the end of digestion for all suspensions. Similarly, only for the pea protein an increase in ACE inhibitory activity was observed. The ACE inhibitory activity of a 2.73 mg/ml sample reached after incubation 80% for *S. cerevisiae's*, 30% for *L. helveticus*' and 40% for the combination's lysed cells. In the whey protein digests no change in degree of proteolysis, nor ACE inhibitory activity, could be detected.

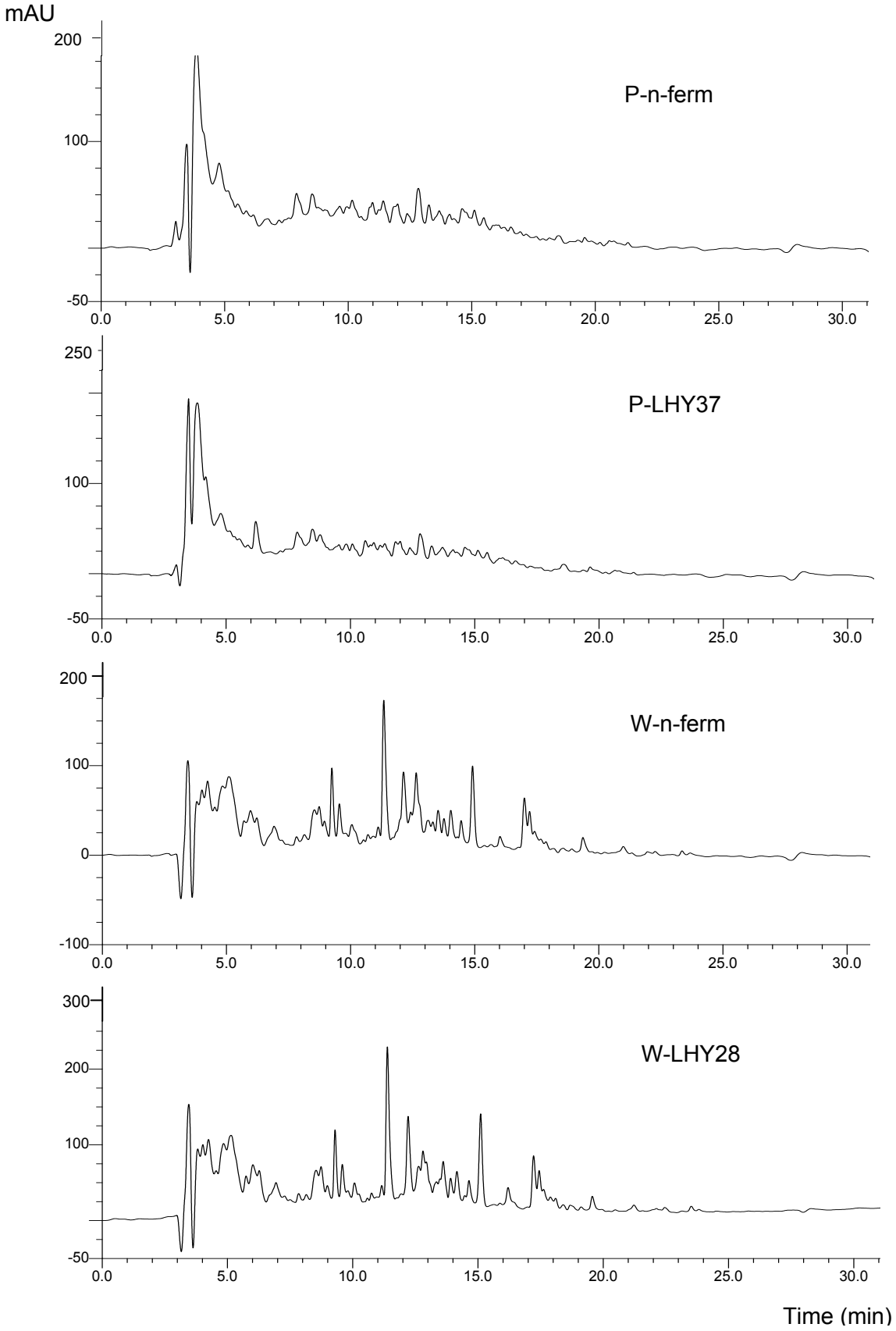

Figure 3. HPLC-profile of the digested non-fermented pea (P-n-ferm) and whey (W-n-ferm) medium, pea medium fermented by *L. helveticus* at 37°C (P-LHY37) and whey medium fermented by *S. cerevisiae* at 28°C (W-LHY28).

DISCUSSION

In this study, pea and whey protein were fermented by lactobacilli and yeast in order to release ACE inhibitory peptides that could lower the blood pressure *in vivo*. Subsequently, the impact of an *in vitro* gastrointestinal digestion on the ACE inhibitory activity was evaluated.

Screening of lactobacilli

From the eight GRAS lactobacilli selected, only *L. helveticus* produced considerable ACE inhibitory activity after fermentation of pea protein medium. *L. helveticus*, whether or not in combination with *S. cerevisiae*, is known for its capacity to form ACE inhibitory peptides from milk proteins (Nakamura *et al.*, 1995a; Yamamoto *et al.*, 1999). Fermented milk prepared by most strains of *L. helveticus* significantly lowers the blood pressure in SHR upon oral administration, while milk fermented by other species of lactic acid bacteria, including a *L. acidophilus* strain, does not display significant antihypertensive effects (Yamamoto *et al.*, 1994a). According to the same study, ACE inhibitory activity in most of the whey fractions of the milk fermented by *L. helveticus* is also higher than in the other fermented milks. Another feature of *L. helveticus* is that it can survive passage through the gastrointestinal tract, where it may act as a probiotic (Shinoda *et al.*, 2001). Nevertheless, ACE inhibitory peptides have been isolated from different dairy products started by various lactic acid bacteria: e.g. *Lactobacillus delbrueckii* subsp. *bulgaricus* SS1, *Lactococcus lactis* subsp. *cremoris* FT4, *Lactobacillus acidophilus*, bifidobacteria, *Streptococcus thermophilus*, … (Gobbetti *et al.*, 2000; Ryhänen *et al.*, 2001; Saito *et al.*, 2000).

Based on the results from the screening and literature, *L. helveticus* and *S. cerevisiae* were used to initiate fermentations in monoculture and in combination on pea and whey protein. The ACE inhibitory activity after fermentation is not a good selection criterion, because the physiological transformations in the human body, which determine the bioavailability of the ACE inhibitory peptides, are not taken into consideration. However, often only the food-processing step is envisaged (Haileselassie *et al.*, 1999; Kim *et al.*, 2001). By incorporation of the *in vitro* gastrointestinal digestion, the measured ACE inhibitory activity relates more closely to the ACE inhibitory activity in the human body. To exert an antihypertensive effect, the peptides also have to pass the intestinal barrier to arrive in the blood in an active form, yet this has been the scope of another paper (Vermeirssen *et al.*, 2002a). From this study, it was hard to make a selection after *in vitro* digestion in the screening, since almost all fermented digests reached maximal ACE inhibitory activity.

Fermentation by *Lactobacillus helveticus* or/and *Saccharomyces cerevisiae*

The lactic acid bacterium *L. helveticus* was metabolically more active at 37°C, which resulted in more lactic acid production and hence a larger pH decrease. Although *L. helveticus* and *S. cerevisiae* have complex nutritional requirements for growth (Hebert *et al.*, 2000; Walker, 1998), both microorganisms were able to grow on the pea and whey protein medium in monoculture at their optimal temperature. The type of protein did not influence their growth, showing that pea and whey protein both could serve as a nitrogen source for *L. helveticus* and *S. cerevisiae*. Furthermore, it cannot be excluded that the (autoclaved) protein isolates contain a minor amount of small peptides and free amino acids.

A positive interaction of the yeast towards the lactic acid bacterium would be translated in the provision of essential growth factors like amino acids and vitamins, CO_2 production or growth stimulation by increasing the pH due to the utilisation of organic acids. However, in the combined fermentations, the yeast did not favour the growth of *L. helveticus*, which could be due to the production of organic acids, antibacterial compounds, ethanol, … or competition for nutrients (Viljoen, 2001). The counts of *S. cerevisiae* on the other hand, decreased slightly in the presence of *L. helveticus* at 37°C; which can either be attributed to the high acid production or the high temperature.

Degree of proteolysis in ferments and digests and protease activity

The increase in degree of proteolysis observed in the ferments with *L. helveticus* was induced by the action of its proteases, possibly supplemented by a slight acid hydrolysis as the pH decreased maximally to 3.5. *L. helveticus* is known to possess a cell-wall-associated protease, capable of forming antihypertensive peptides from casein (Yamamoto *et al.*, 1994b), and several peptidases (Christensen *et al.*, 1999). However, the protease activity assay found a higher activity in the supernatant than in the cell precipitate, indicating that the cell-wall-associated protease has been released from the cell wall or that other secreted proteases played a major role. Also, the peptide rich propagation medium may inhibit the synthesis of the cell-wall-associated protease in *L. helveticus* (Hebert *et al.*, 2000). The total potential protease activity of *S. cerevisiae* was similar to that of *L. helveticus*, but this did not induce an increase in degree of proteolysis after fermentation. Generally, *S. cerevisiae* has no extracellular proteases or peptidases to cleave oligopeptides or proteins in the medium, only some specialised strains have (Walker, 1998). However, it seems that the strain used in this study did possess some extracellular protease activity. A non-significant increase and even decrease in degree of proteolysis by the yeast can be explained by the consumption of peptides and amino acids present in the medium and by an increase in total protein content

due to yeast growth. The gastrointestinal proteases drastically incremented the degree of proteolysis, which simulated well the conditions during physiological digestion. The specificity and purity of pepsin, trypsin and α-chymotrypsin led to the formation of numerous peptides and amino acids. The pea protein was more susceptible to hydrolysis than the whey protein.

ACE inhibitory activity in ferments and digests

Comparing the ACE inhibitory activity of the whey protein at the start of the fermentations with that at the start of the digestion by intracellular proteases revealed that autoclaving had a major impact on the ACE inhibitory activity of the heat labile whey protein. This was also to a lesser extent observed for the ACE inhibitory activity of pea and was reflected in the degree of proteolysis for both proteins. This may be caused by heat and by the presence of natural proteases in the (whey) protein isolate, which are initially activated by the temperature increase during autoclaving (Huyghebaert, 1999). Yet, no proteolysis is observed during heat treatment of whey protein isolate at 85°C for 30 min at pH 4.6 (Mutilangi *et al.*, 1995). The higher initial degree of proteolysis of whey compared to pea protein can be explained by a higher concentration of peptides, like CMP, in this protein isolate.

In vitro gastrointestinal digestion released significant ACE inhibitory activity from pea and whey protein medium and ferments. However, despite the growth of the microorganisms, the increase in degree of proteolysis and the increase in ACE inhibitory activity after fermentation, the fermented samples did not show higher ACE inhibitory activity after *in vitro* digestion than the non-fermented samples. The fact that fermentations are ineffective in producing ACE inhibitory activity has been reported in literature. Pihlanto-Leppällä *et al.* (1998) observed ACE inhibitory activity in whey and casein fermented by different lactic acid starters only after digestion by trypsin and pepsin. They attribute their results to the low proteolytic activity of the starters or the specificity of the enzymes in lactic acid bacteria. In a study where milk, either supplemented with 5% (w/w) sodium caseinate or whey protein isolate, is fermented by two *L. helveticus* strains for 24 h under constant stirring and CO_2 circulation at 42°C and pH 6, the caseinate-enriched milk allows better growth conditions and shows a higher degree of proteolysis and ACE inhibitory activity than the milk containing whey protein isolate (Leclerc *et al.*, 2002). This suggests that casein is a better substrate than whey proteins for the extracellular proteinases of lactic acid bacteria. This may be another reason why there was no high ACE inhibitory activity obtained after fermentation of pea or whey protein. The formation of bioactive peptides by lactic acid bacteria seems to be a rare event and is recently being debated (Meisel and Bockelmann, 1999).

The IC_{50} values not only confirmed this observation, but even pointed out that fermentation may sometimes adversely affect the ACE inhibitory activity as the non-fermented whey protein digest showed the highest ACE inhibitory activity. The tendency towards higher IC_{50} values in the fermented digests can be enlightened by the hypothesis that the microbial enzymes splice within the sequence of bioactive peptides in the food protein, thereby preventing the gastrointestinal proteases to release them. Therefore, there exists an optimal degree of hydrolysis, above which more ACE inhibitory peptides are degraded than new peptides are formed, decreasing the overall ACE inhibitory activity (Mullally *et al.*, 1997a; Pedroche *et al.*, 2002). Hence, no direct relationship between the degree of proteolysis and the ACE inhibitory activity is possible, especially in the later stages of hydrolysis. After fermentation of the whey but not the pea protein, some correlation between the degree of proteolysis and the ACE inhibitory activity was observed. After fermentation and digestion, however, the whey samples fermented by *S. cerevisiae* at 28°C, which showed the lowest degree of proteolysis and ACE inhibitory activity after fermentation, exhibited the lowest IC_{50} values and hence the highest ACE inhibitory activity. This further illustrates our hypothesis. The IC_{50} values of the digests ranged from 0.072 to 0.183 mg/ml, which are slightly more active than the values reported for whey digests by gastrointestinal proteases of 0.345 to 1.733 mg/ml (Pihlanto-Leppälä *et al.*, 2000). Differences in assay conditions make it somewhat difficult to compare these values. The tendency towards lower IC_{50} values for the whey compared to the pea digests was due to the intrinsic amino acid sequence of the protein.

HPLC profiles of the digests

The HPLC chromatograms of the peptide fraction with MWCO < 3000 Da, did not differ between fermented and non-fermented digests. The type of protein and the digestion step determined the HPLC profile and hence the formation of small peptides with possible ACE inhibitory effects. Fermentation had only minor influence on the small peptide profile. Matar *et al.* (1996), however, concludes that there is a major impact of milk fermentation by *L. helveticus* on subsequent gastrointestinal digestion. The HPLC elution profiles of that study show that fermentation may contribute to the formation of new peptides during *in vitro* digestion, provided that the pH is controlled at 6.0 during the fermentation, which favours the growth and proteolytic activity of *L. helveticus*. Nevertheless, in other fermentation studies with *L. helveticus* and other lactic acid bacteria that are effective in producing ACE inhibitory peptides, pH is never controlled (Gobbetti *et al.*, 2000; Nakamura *et al.*, 1995a; Yamamoto *et al.*, 1999).

Contribution of intracellular proteases

In the case of the pea protein, the preliminary study on the production of ACE inhibitory activity from lysed cells pointed to the importance of the intracellular proteases from *S. cerevisiae*. Lysis of the cells made these proteases readily available for cleaving the bioactive peptide sequences from the food proteins, leading to an increased ACE inhibitory activity. Intracellular proteases and peptidases of both *L. helveticus* and *S. cerevisiae* will most likely contribute to degradation of proteins after cell lysis during fermentation (Jones, 1990; Law and Haandrikman, 1997; Meisel and Bockelmann, 1999). This may both positively or negatively affect the ACE inhibitory activity.

High ACE inhibitory activity is identified in skimmed milk digested with cell-free extract of *S. cerevisiae* and the hydrolysate obtained with the purified protease B shows an IC_{50} of 0.42 mg protein/ml (Roy *et al.*, 2000). Another vacuolar protease of *S. cerevisiae*, carboxypeptidase Y, is ineffective in releasing the potent ACE inhibitor Val-His-Leu-Pro-Pro from γ-zein (Maruyama *et al.*, 1989). An extract of autologous *Lactobacillus casei* cell lysate demonstrates an antihypertensive effect in hypertensive patients, but this is due to the presence of polysaccharide-glycopeptide complexes (Nakajima *et al.*, 1995).

In conclusion, we were able to produce ACE inhibitory active digests from pea and whey protein. Nevertheless, a fermentation step by *L. helveticus* or/and *S. cerevisiae* did not improve the release of ACE inhibitory activity after *in vitro* gastrointestinal digestion.

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CHAPTER 5

ACE INHIBITORY *IN VITRO* GASTROINTESTINAL DIGESTS: FROM BATCH TO SEMI-CONTINUOUS MODEL

Redrafted after VERMEIRSSEN, V., VAN CAMP, J., DEVOS, L. & VERSTRAETE, W. (2003b). Release of angiotensin I converting enzyme (ACE) inhibitory activity during *in vitro* gastrointestinal digestion: from batch experiment to semi-continuous model. *Journal of Agricultural and Food Chemistry. Submitted.*

Chapter 5

ACE inhibitory in vitro gastrointestinal digests: from batch experiment to semi-continuous model

ABSTRACT

Gastrointestinal digestion is of major importance in the bioavailability of angiotensin I converting enzyme (ACE) inhibitory peptides, bioactive peptides with possible antihypertensive effects. In this study, the conditions of *in vitro* gastrointestinal digestion leading to the formation and degradation of ACE inhibitory peptides were investigated for pea and whey protein. In batch experiments, the digestion simulating the physiological conditions sufficed to achieve the highest ACE inhibitory activity, with IC_{50} values of 0.076 mg/ml for pea and 0.048 mg/ml for whey protein. The degree of proteolysis did not correlate with the ACE inhibitory activity and was always higher for pea than for whey. In a semi-continuous reactor model of gastrointestinal digestion (pre-SHIME), response surface methodology studied the influence of temperature, incubation time in the stomach and incubation time in the small intestine phase on the ACE inhibitory activity and the degree of proteolysis. For the pea protein a linear model for the degree of proteolysis and a quadratic model for the ACE inhibitory activity could be constituted. Within the model, maximal degree of proteolysis was observed at the highest temperature and the longest incubation time in the small intestine phase; while maximal ACE inhibitory activity was obtained at the longest incubation times in the stomach and the small intestine phase. These results show that ACE inhibitory activity of pea and whey hydrolysates can be controlled by the conditions of the *in vitro* gastrointestinal digestion.

Keywords: ACE inhibitory peptides, pea protein, whey protein, response surface methodology

INTRODUCTION

Angiotensin I converting enzyme (ACE, EC 3.4.15.1) inhibitory peptides are food derived bioactive peptides with possible antihypertensive effects *in vivo* (Fitzgerald and Meisel, 2000). A high blood pressure is a major risk for cardiovascular disease, one of the most important causes of mortality in the developed world. In a recent study performed in 5 European countries, 34% of the adult population had a blood pressure higher than 140/90 mmHg, the treatment of which contributes substantially to health care costs (Hansson *et al.*, 2002). Recently, foods containing ACE inhibitory peptides have shown to be effective in both the prevention and treatment of hypertension (Sipola *et al.*, 2002; Takano, 1998).

Research on ACE inhibitory peptides has mainly concentrated on milk proteins, but vegetable and other animal proteins have been studied as well (Yamamoto, 1997). To our knowledge, pea protein has not been the subject of study for ACE inhibitory activity yet. In a previous paper, we observed a large impact of *in vitro* gastrointestinal digestion on the formation of ACE inhibitory activity from pea and whey protein (Vermeirssen *et al.*, 2003a). The scope of this paper was to further investigate the conditions of *in vitro* gastrointestinal digestion leading to the formation and/or degradation of ACE inhibitory peptides. Firstly, this is of physiological importance, since, upon oral administration, these bioactive peptides have to reach the blood stream in an active form to exert an antihypertensive effect (Masuda *et al.*, 1996). Gastrointestinal digestion and transport are the major barriers in the bioavailability of ACE inhibitory peptides (Pihlanto-Leppälä, 2001). Secondly, digestion by gastrointestinal proteases can be used as a production process for ACE inhibitory peptides, with the advantage that the formed peptides will resist the physiological digestion after oral intake (Matsui *et al.*, 2002).

Few studies have been performed on the conditions necessary during digestion to release ACE inhibitory activity. Some hydrolysis of the proteins is required in order to free the bioactive peptide sequences. At some point during hydrolysis however, no further increase in ACE inhibitory activity is observed (Mullally *et al.*, 1997a). Meisel *et al.* (1997) reported that the ACE inhibitory activity in ripened cheese increases during cheese maturation, but decreases when the proteolysis exceeds a certain level. At this point the degradation of bioactive peptides starts to dominate the formation of new ones. Digestion of bovine skin gelatine independently by different proteases at incubation times up to 24 h also demonstrated an optimal incubation time for maximal ACE inhibitory activity (Kim *et al.*, 2001). Hence, there seems to be an optimal and not always maximal hydrolysis for maximal ACE inhibitory activity. Moreover, the specificity of the enzymes plays a major role in the formation of ACE inhibitory peptides (Mullally *et al.*, 1997a; Vermeirssen *et al.*, 2003a). ACE

inhibitory peptides are most commonly produced by trypsin (EC 3.4.21.4) (Pihlanto-Leppälä, 2001). This readily available enzyme forms peptides with a terminal lysine or arginine, the latter being reported in structure-activity studies of ACE inhibitory peptides (Fitzgerald and Meisel, 2000). The digestion of α-lactalbumin and β-lactoglobulin by pepsin (EC 3.4.23.1), trypsin, α-chymotrypsin (EC 3.4.21.1), pancreatin, elastase (EC 3.4.21.36) or carboxypeptidase A (EC 3.4.17.1) and B (EC 3.4.17.2) alone and in combination, revealed that trypsin is necessary to release high ACE inhibitory activity from whey protein. The gastrointestinal protease elastase, on the other hand, is associated with a low production of ACE inhibitory peptides from α-lactalbumin and β-lactoglobulin (Mullally *et al.*, 1997a; Pihlanto-Leppälä *et al.*, 2000).

The physiological conditions of the gastrointestinal protein digestion are more or less known. The gastric pH is between 1.5 and 2 during fasting and it can increase up to 5 after ingestion of a meal due to the diluting and buffering effect of the food components (Charman *et al.*, 1997). Proteins are cleaved in the stomach by the endopeptidase pepsin with rather broad substrate specificity and an optimal pH in the range of 1 to 2 (Ganapathy and Leibach, 1999). The fasting pH of the overall small intestine is situated around 6.5, while for the postprandial pH this is around 5.5 (Charman *et al.*, 1997). In the duodenum, the endopeptidases trypsin, chymotrypsin and elastase, and the carboxypeptidases A and B continue the splicing of the polypeptide chain at more alkaline pH with an optimal activity in the range 7 to 8. At the brush border membrane, the oligopeptides are further cleaved by amino-, di- and endopeptidases, resulting in a mixture of amino acids and small peptides which can be absorbed by the enterocytes (Ganapathy and Leibach, 1999). The half-emptying time for the stomach is 0.5 to 3 h for fed conditions, while the residence time in the duodenum and jejunum is 2 to 2.75 h and in the ileum 5 to 7 h (Oomen *et al.*, 2002).

In our study, digestion was first simulated in batch, where the enzyme over substrate ratio was set at 1 over 250 (w/w) (Gauthier *et al.*, 1986). A non-optimal, physiological and prolonged optimal gastrointestinal digestion, varying in pH and residence time in stomach and small intestine, were compared for the formation of ACE inhibitory activity, degree of proteolysis and HPLC profile. Subsequently, a semi-continuous model based on the batch physiological digestion, was developed. In this reactor, the influence of temperature, incubation time in stomach and small intestine phase on the formation of ACE inhibitory activity and degree of proteolysis was investigated by means of an experimental design.

MATERIALS AND METHODS

Products

The pea protein isolate Pisane® HD (90% protein on dry matter) and the whey protein isolate Lacprodan® DI-9213 (minimal 90% protein on dry matter) were obtained from Cosucra SA (Fontenoy, Belgium) and Acatris Belgium NV (Londerzeel, Belgium), respectively. Pepsin (P 6887), trypsin (T 1426), α-chymotrypsin (C 4129), trichloroacetic acid solution (TCA) (490-10), ACE reagent (305-10), ACE control-E (A 7040), 100% (w/w) trifluoroacetic acid solution (TFA) (30,203-1) were purchased from Sigma-Aldrich (St.-Louis, MO, USA). Non-specified products were analytical grade from VWR International (Zaventem, Belgium).

Batch gastrointestinal digestion

A 100 ml 4% (w/v) protein isolate solution was brought to the desired pH for the stomach digestion with 1 N and 10 N HCl and NaOH under rigorous mixing. Pepsin was added in an E/S of 1/250 (w/w) after which the incubation at 37°C on a shaker started. After a defined residence time, the pH was set at the desired value for small intestine digestion and trypsin and α-chymotrypsin were supplemented both in an E/S of 1/250 (w/w). Then the solution was again incubated at 37°C. When samples were taken at the start and the end of digestion, the pH was adjusted to 5. As this is a pH near the iso-electrical point for both proteins (pea: pH 4.5, whey: pH 4 to 5), a standardised and clear separation was obtained by subsequent centrifugation. Incubation time and pH in stomach and small intestine phase varied in the three types of *in vitro* gastrointestinal digestion: the non-optimal, physiological and prolonged optimal digestion (Table 1).

Table 1. pH and incubation time (h) in stomach and small intestine phase for the non-optimal, physiological and prolonged optimal digestion.

Type of digestion	Stomach		Small intestine	
	pH	Incubation time	pH	Incubation time
Non-optimal	4	0.5	5	0.5
Physiological	2	2	6.5	2.5
Prolonged optimal	2	4	7	4.5

For the preliminary experiments preceding the experimental design, other protein concentrations (2 and 8% (w/v)) and enzyme over substrate ratios (1/50, 1/100, 1/400, 1/1000) were applied in the batch gastrointestinal digestion.

Experimental design in a semi-continuous model for gastrointestinal digestion

Figure 1 shows the reactor set-up for the semi-continuous digestion. This model was called the pre-SHIME, because it can precede the SHIME, the Simulator of the Human Intestinal Microbial Ecosystem (Molly *et al.*, 1993). By means of double jacketed vessels and a warm water bath, the content of the vessels was kept at a defined temperature. The pH in the two vessels was controlled by a pH stat (Consort R305, Turnhout, Belgium) and the content was mixed by magnetic stirring. A peristaltic pump brought 200 ml 4% (w/v) protein solution in the first reactor vessel that simulated the stomach. The pH was adjusted to 2 (lower and upper limits: 1.85 and 2.15) with 1 N HCl and NaOH. Pepsin was supplemented in an E/S of 1/250 (w/w). After the stomach phase the solution was pumped to the second reactor vessel, where the pH was set at 6.5 (lower and upper limits: 6.35 and 6.65). Subsequently, trypsin and α-chymotrypsin were added in an E/S of 1/250 (w/w) and the solution was incubated for the small intestine phase. At the end of the *in vitro* digestion the hydrolysate was pumped to an erlenmeyer flask and brought to pH 5. Samples for analyses were taken.

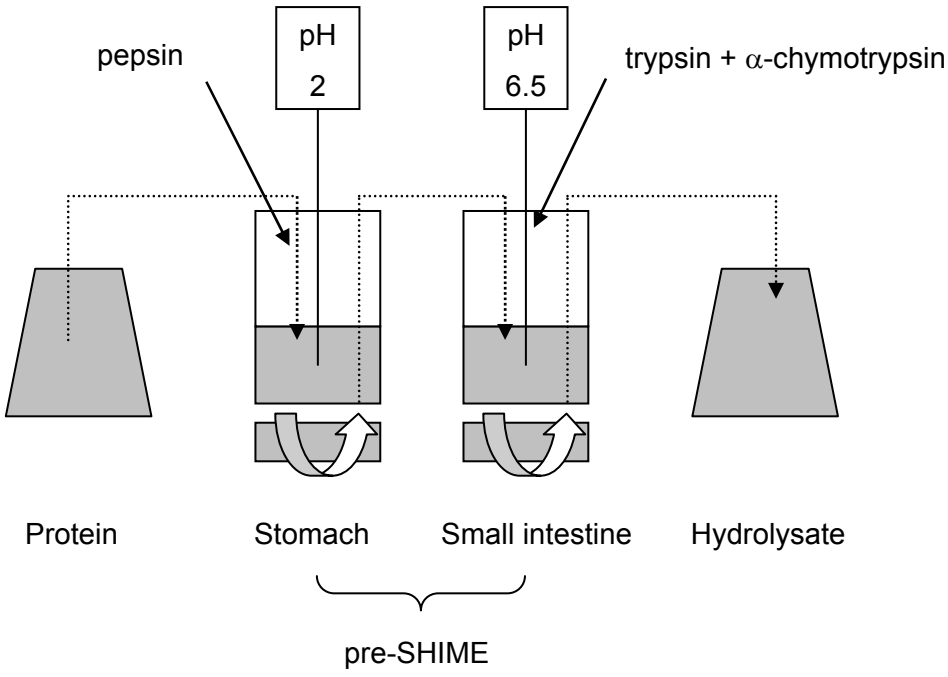

Figure 1. Experimental set up for the semi-continuous digestion.

The experimental design was created and analysed by Design-Expert 6.0.3 (Stat-Ease, Minneapolis, USA). Response Surface Methodology linked the digestion parameters temperature, incubation time in stomach phase and incubation time in small intestine phase (3 factors) via a polynome to the degree of proteolysis and the 50% ACE inhibitory concentration (IC_{50}) (2 responses). A central composite design was chosen, where the factors vary round a central point, here the conditions of the physiological digestion (Table 1, Figure 2). This design contains 5 levels for each digestion parameter, coded by -α, -1, 0, +1 and +α (Table 2). In this way, the factors are standardised and vary in the same range, which facilitated the interpretation of the effects and interactions.

Table 2. The different factors of the central composite design with their codes and values.

Factor	Code				
	-α	-1	0	+1	+α
Temperature (°C)	18.5	26.0	37.0	48.0	55.5
Incubation time stomach (min)	19	60	120	180	221
Incubation time small intestine (min)	0	60	150	240	301

The design exists of three experiment subsets. Firstly, at the central point, all the factors are at level 0. Secondly, the factorial points have all combinations of the factors set at the levels -1 and +1 and indicate the range where the design is valuable. These points give information about the linear effects and the interaction effects. Thirdly, for the axial points, all factors are set at level 0, except for one, which adopts the levels –α or +α, an extreme value. These points determine the estimation of the quadratic effects. The value of α was chosen so the design was rotatable, which guaranteed that the precision of the estimated values (standard error) is only influenced by the distance to the central point and not by the direction. The final experimental design consisted of 20 experiments for both proteins: 6 repetitions of the central point, 8 factorial points and 6 axial points. Based on the repetitions of the central point, the experimental error was estimated.

Degree of proteolysis

The degree of proteolysis was determined as in Chapter 4.

ACE inhibitory activity

Samples for the determination of ACE inhibitory activity were treated as described in Chapter 4 and analysed by the ACE inhibition assay 3 in Chapter 2.

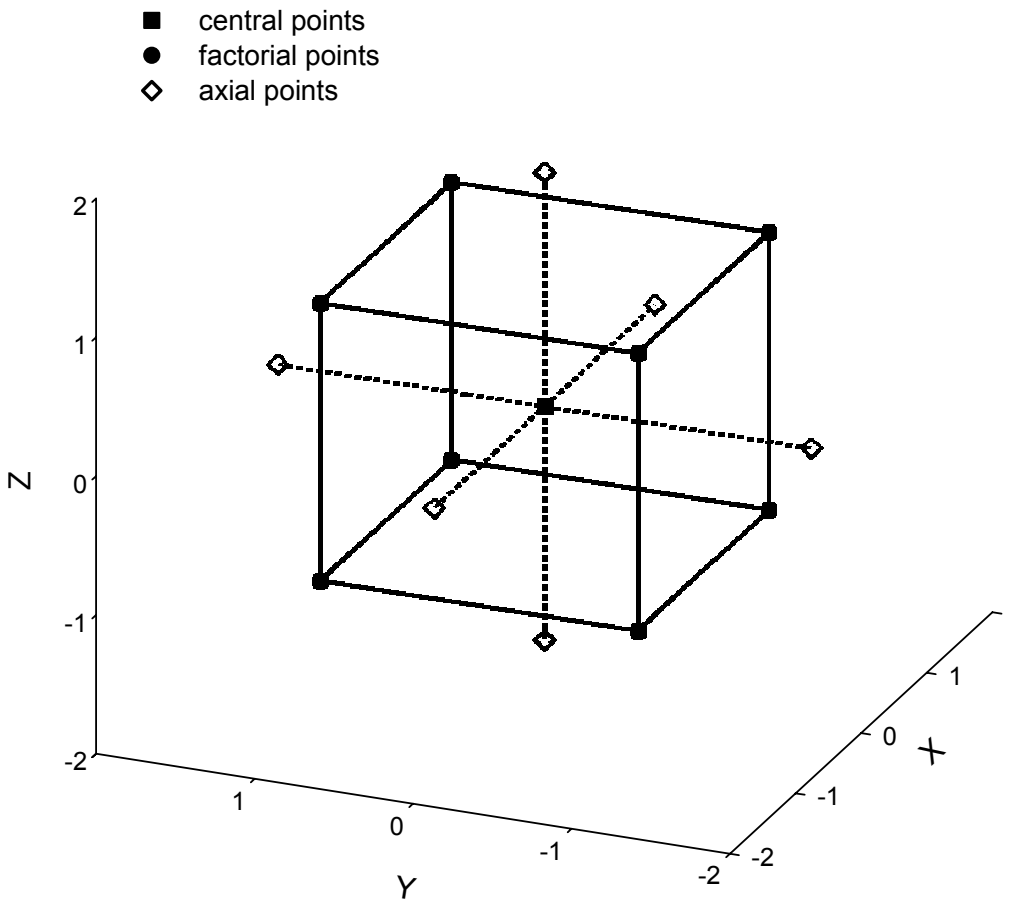

Figure 2. Central composite design for three factors: position of factorial and axial points relative to central points.

HPLC

Samples for the HPLC analysis were treated as for the ACE inhibition assay. Ten milligram lyophilised sample was dissolved in 1 ml milli-Q water and 20 µl of this solution was analysed by RP-HPLC on a Prosphere 300 Å C18 column (250 x 4.6 mm, 5 µm) (Alltech Associates, Deerfield, IL, USA) and a Dionex (Sunnyvale, CA, USA) HPLC with an autosampler ASI-100, pump series P580, STH585 column oven, UV-VIS detector UVD340S operating at 210 nm and Chromeleon 6.0 software. Elution was at 25°C with a flow rate of 1 ml/min with solvents A (H_2O + 0.1% (w/v) TFA) and B (acetonitrile + 0.85% (w/v) TFA). Whey was analysed by a linear gradient from 90% solvent A to 50% solvent B in 30 min, again to 90% solvent A in the next 20 min and remaining at 90% solvent A in the last 10 min. For pea a linear gradient from 95% solvent A to 30% solvent B in 30 min was applied. Subsequently, the gradient went linearly up to 95% solvent A the next 20 min and remained at 95% solvent A in the last 10 min. A control solution containing 16 mg of pepsin, trypsin and α-chymotrypsin in 100 ml MilliQ, centrifuged for 15 min at 10 000 g, was also eluted by

the HPLC programs for pea and whey respectively. Only an injection peak could be observed on these chromatograms.

Statistical analysis

All values are reported as mean ± standard error (SE) of the mean (n_{min} = 3). For the comparison of the three *in vitro* digestions, significant differences in type of protein (p_{prot}) and type of digestion (p_{digest}) were assigned by means of the General Linear Model Univariate Analysis of Variance procedure (SPSS 11.0.1, Chicago, Illinois, USA). To exclude the initial effect of the protein itself, the statistical analysis was performed on the changes in degree of proteolysis and ACE inhibitory activity during digestion. When there was a significant type of protein × type of digestion interaction, for both proteins a Oneway ANOVA analysis was carried out by type of digestion. All data used in the variance analysis met the homogeneity of variance requirement assessed by Levene's test. Indication of subgroups in type of digestion was done by Tukey post hoc test ($p < 0.05$). Differences between the digestion in the semi-continuous reactor model and the physiological digestion in batch were assigned by an independent Student t-test. A hierarchical cluster analysis by average linkage and with the Pearson correlation coefficient as measure of inequality, was performed on the relative squares of the peaks for different intervals of retention times of the digests HPLC chromatograms. An independent Student t-test compared the log IC_{50} values of the preliminary batch experiments of the experimental design and the batch physiological digestion.

RESULTS

Comparison of three different *in vitro* gastrointestinal digestions

Before digestion, the pea protein solution showed a lower degree of proteolysis than whey, respectively 2.4 ± 0.4% and 12.9 ± 0.7% ($p < 0.001$), while the ACE inhibitory activity of both proteins did not differ and was 10 ± 0.7% and 15 ± 2%, respectively ($p = 0.083$). IC_{50} values were 16 mg/ml for pea and 18 mg/ml for whey protein.

To investigate the conditions during gastrointestinal digestion leading to the release of ACE inhibitory activity, we compared three different *in vitro* digestions (Table 1). The non-optimal digestion was carried out at non-optimal pH in stomach and small intestine phase for short incubation times, minimizing protein hydrolysis. The physiological digestion simulated the *in vivo* conditions of protein digestion. The prolonged optimal digestion combined optimal pH in stomach and small intestine phase with long incubation times in order to get ideal conditions for protein hydrolysis.

The degree of proteolysis after the three *in vitro* digestions is depicted in Figure 3. The statistical analysis, however, was performed on the change in degree of proteolysis during digestion, to exclude the initial degree of proteolysis of the protein itself. For both proteins, the increase in degree of proteolysis was significantly different between the three digestions (Oneway Anova, $p_{digest} < 0.001$). For all digestions, pea showed a higher increase in degree of proteolysis than whey. For the whey protein, the degree of proteolysis increased proportionally between the three digestion types, while for the pea protein, the prolonged optimal digestion contributed only a little more to the degree of proteolysis than did the physiological digestion ($p_{interaction} = 0.003$). In the physiological digests, the degree of proteolysis amounted to 72 ± 1% for pea and 58 ± 2% for whey.

The increase in percent ACE inhibitory activity did not vary significantly between the three *in vitro* gastrointestinal digestions ($p_{digest} = 0.078$) and amounted to 89.8 ± 0.3% for pea and 83.8 ± 1.2 % for whey digest respectively ($p_{prot} < 0.001$). As the maximal level of 100% ACE inhibitory activity was reached after all digestions, the (log) IC_{50} value or 50% inhibitory concentration was determined (Figure 3). A higher IC_{50} indicated a lower ACE inhibitory activity. The three digestions had a different effect on the log IC_{50} value for the pea in comparison to the whey protein ($p_{interaction} < 0.001$). For pea, the only significant difference was found between the non-optimal and the physiological digestion, where the IC_{50} of the first was double the one of the latter (Oneway Anova, $p_{digest} = 0.015$).

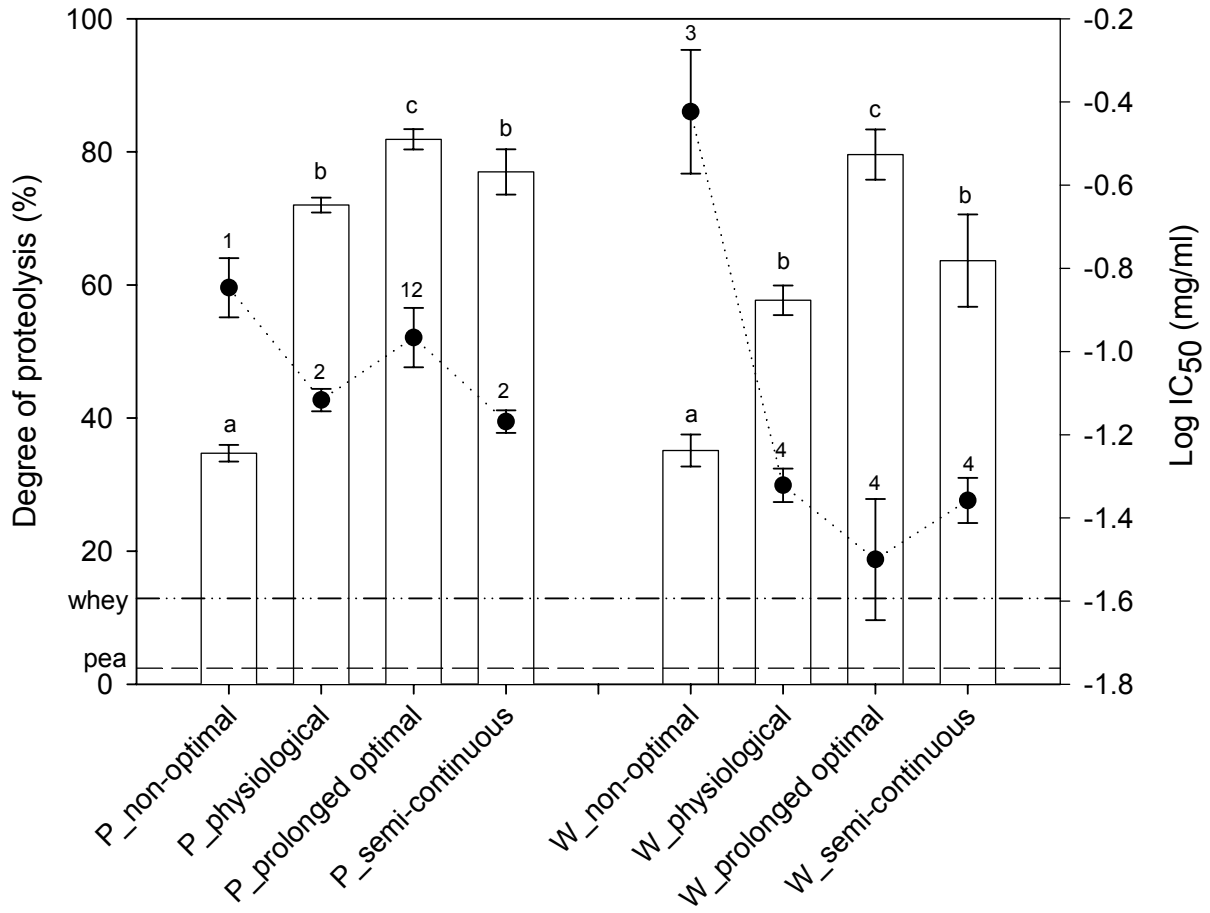

Figure 3. Degree of proteolysis (%) (bars, a, b, …) and log IC_{50} (mg/ml) (bullets, 1, 2, …) after the non-optimal, physiological and prolonged optimal digestion and the semi-continuous digestion of pea (P) and whey (W) protein (n_{min} = 3). Straight, dashed lines indicate the initial degree of proteolysis of the pea and whey protein. Different letters and numbers indicate significant differences ($p < 0.05$). The semi-continuous digestion was compared to the physiological digestion by means of an independent samples t-test.

For the whey, on the other hand, a relatively high IC_{50} was obtained after non-optimal digestion, which displayed a tenfold difference from the IC_{50} of the physiological and prolonged optimal digestion (Oneway Anova, $p_{digest} < 0.001$). IC_{50} values as low as 0.076 mg/ml for the physiological digestion of pea and 0.032 mg/ml for the prolonged optimal digestion of whey protein were observed (Table 3).

The soluble fraction of the non-digested proteins and the digests of both proteins after non-optimal, physiological and prolonged optimal digestion were eluted by RP-HPLC (Figure 4a and b). The chromatograms clearly differentiated both proteins. For the pea protein, the chromatograms of the prolonged optimal digests had remarkably higher initial

peaks compared to the ones of the non-optimal and physiological digests, while apart from that no major differences were noticed. For the whey protein, it is clearly shown that the β-lactoglobulin peak was only substantially degraded in the prolonged optimal digests (this peak was confirmed by elution of pure β-lactoglobulin). Moreover, in the non-optimal digest a remainder of another whey protein at a retention time of 30 min is present. From the non-optimal to the physiological and to the prolonged optimal digestion, more intense peptide peaks were observed, as the degree of proteolysis increased. The relative squares of the peaks for different intervals of retention times clustered separately for the pea and whey digests (Figure 5). Furthermore, the data of the different pea digests were more closely related with each other than the data of the different digestions of whey.

Table 3. IC_{50} (mg/ml) of pea and whey protein and after non-optimal, physiological, prolonged optimal digestion and after digestion in the semi-continuous reactor.

Digestion type	Pea	Whey
None	16	18
Non-optimal	0.142	0.377
Physiological	**0.076**	**0.048**
Prolonged optimal	0.108	0.032
Semi-continuous	0.068	0.044

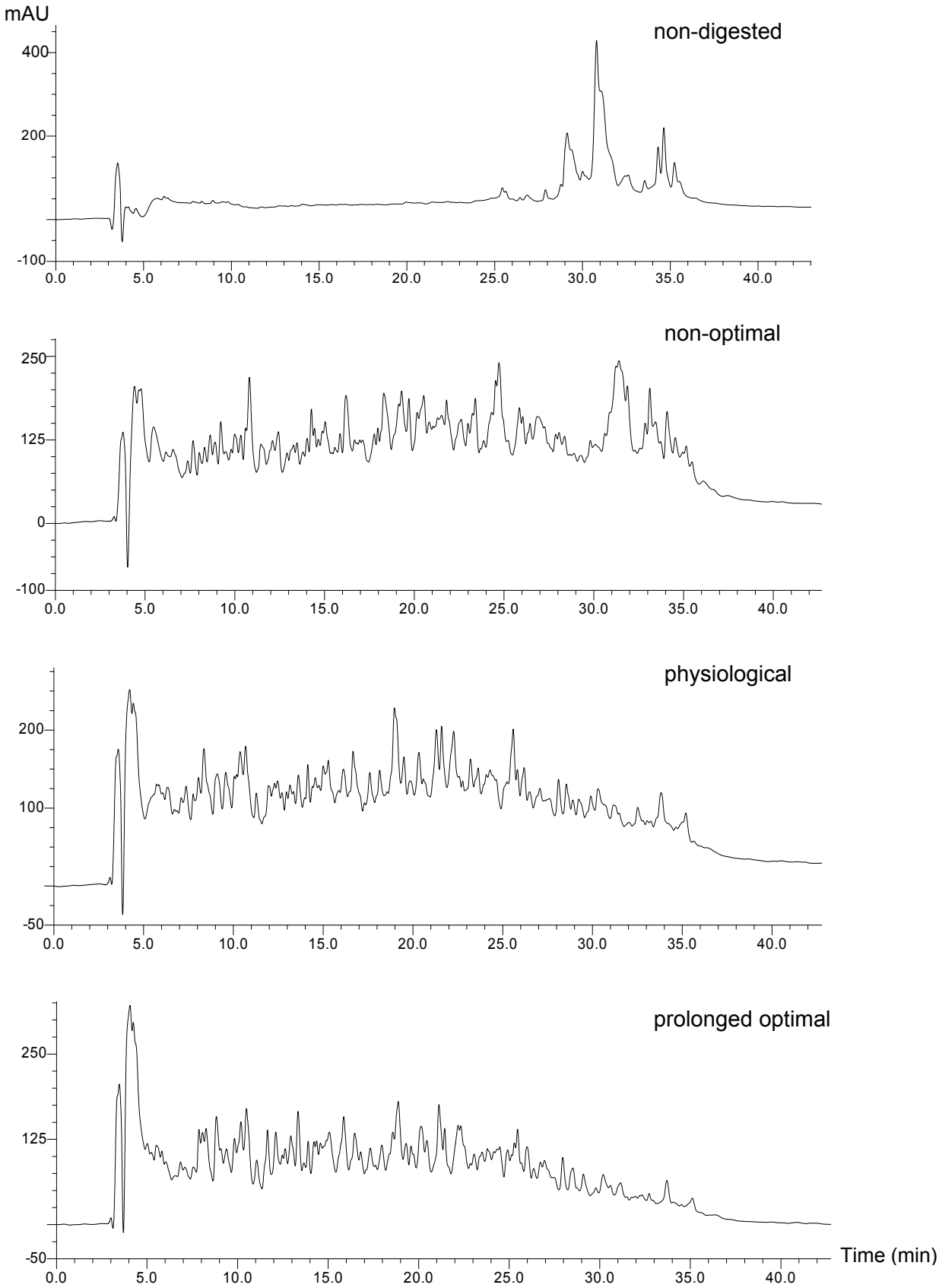

Figure 4a. HPLC chromatograms of the soluble fractions of the non-digested PEA protein and after a non-optimal, physiological and prolonged optimal digestion.

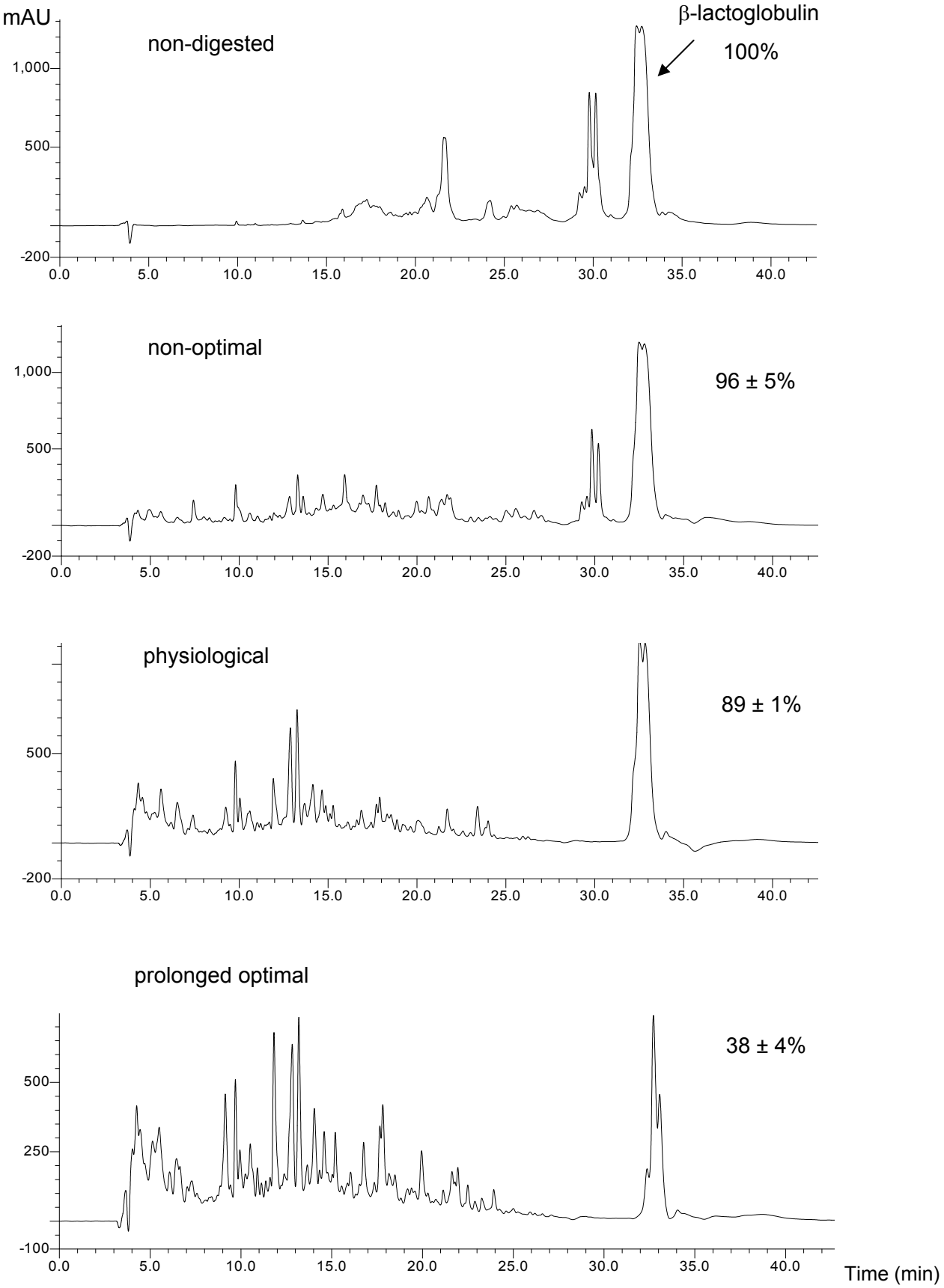

Figure 4b. HPLC chromatograms of the soluble fractions of the non-digested WHEY protein and after a non-optimal, physiological and prolonged optimal digestion. The recovery of β-lactoglobulin is also indicated (n_{min} = 3).

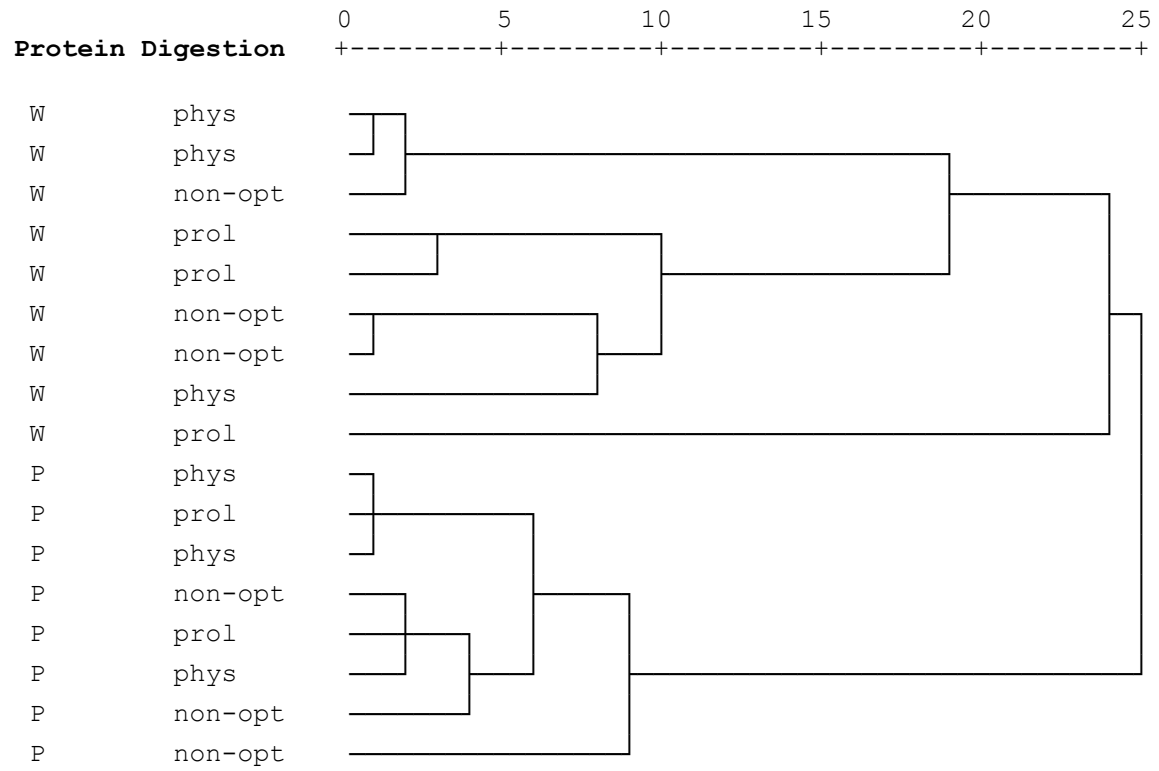

Figure 5. Dendrogram of the hierarchical cluster analysis using average linkage and Pearson correlation coefficient of the relative squares of the chromatogram peaks for different intervals of retention times (W = whey protein, P = pea protein, non-opt = non-optimal, phys = physiological, prol = prolonged optimal digestion).

Experimental design in a semi-continuous reactor model

When the physiological digestion was simulated in the semi-continuous reactor, no significant differences in change in degree of proteolysis (pea, $p = 0.13$, whey, $p = 0.38$) and log IC_{50} value (pea, $p = 0.20$, whey, $p = 0.60$) were observed in comparison to the batch physiological digestion (Figure 3, Table 3). From the HPLC chromatogram, it was observed that 65% of β-lactoglobulin was recovered (data not shown).

First some preliminary experiments were designed in batch to study the influence of protein concentration and enzyme over substrate ratio on the formation of ACE inhibitory activity from pea and whey protein (Table 4). No significant differences between the log IC_{50} values of the whey digests with 2%, 4% or 8% (w/v) protein concentration were found. A 2% (w/v) pea protein solution gave a significantly lower ACE inhibitory activity compared to the other pea protein concentrations. Although the log IC_{50} values of digests with a higher enzyme over substrate ratio tended to be lower for pea protein, the differences were non-significant. The influence of the incubation time in stomach and small intestine phase was already suggested in the previous experiments. In addition, it is known that pepsin A has an

optimal activity at 42°C (Ryle, 1984), while for trypsin this is at 45°C (Venkatesk and Sundaram, 1998). The optimal hydrolysis temperature for α-chymotrypsin is 50°C (Angelo *et al.*, 1982).

Table 4. IC_{50} (mg/ml) of the preliminary experiments for the experimental design *(batch physiological digestion)*. The other conditions were the same as during an *in vitro* physiological digestion.

Condition tested	Pea	Whey
2% (w/v) protein	0.191***	0.047
4% (w/v) protein[§]	*0.076*	*0.048*
8% (w/v) protein	0.079	0.046
E/S = 1/1000	0.082	/
E/S = 1/400	0.075	/
E/S = 1/250	*0.064*	/
E/S = 1/100	0.057	/
E/S = 1/50	0.052	/

/ Data not available.
Statistical analysis performed on the log IC_{50}: *p < 0.05, **p < 0.01, ***p < 0.001 (n = 9).
[§]The values were compared to the mean value of all batch physiological digestions.

Based on these data, temperature and incubation time in stomach and small intestine phase were chosen as parameters in the experimental design in the semi-continuous reactor. The aim of this design was to establish, for both pea and whey protein, a relation between the responses, the degree of proteolysis and the ACE inhibitory activity, expressed as log IC_{50} value, and the variables, temperature (from 26 to 48°C), incubation time in the stomach phase (from 1 to 3 h) and incubation time in the small intestine phase (from 1 to 4 h) within a certain degree of freedom around the physiological digestion parameters (37°C, 2 h and 2.5 h) (Table 2). As the IC_{50} was calculated by means of the four parametric logistic model, only the log IC_{50} is normally distributed. Moreover, the Box Cox plot for power transformation in the Design Expert analysis program indicated that a log transformation for the IC_{50} data was necessary.

The statistic properties of the data points in the experimental design were investigated for the two responses for both proteins (Table 5). Both for the degree of proteolysis and the log IC_{50} value of the pea digests, the range of all data points included the range of the central points, the only replicates in the design. Moreover, the standard error on the average of the central points was smaller than the one on the average of all data points. This indicated a

good reproducibility of the semi-continuous digestion of the pea protein. Hence, the large standard error on the average of all pea digests and the large range were probably not due to experimental error, but to variation in the process parameters. This justified the creation of a response surface model for the pea protein in the two responses. For the whey protein however, the central points showed a rather large variation for both responses, which indicated a poor reproducibility of the semi-continuous digestion of the whey protein. Since the standard error on the average of all samples was about as large as the one on the average of the central points, it could not be explained by a variation in process parameters and was merely due to experimental error. As a result, neither for the degree of proteolysis nor for the log IC_{50} of the whey protein, a significant model could be established.

Table 5. Average ± standard error and range of all points in comparison to the central points for the degree of proteolysis (%) and the log IC_{50} (mg/ml) in the central composite design (n = 20).

		Degree of proteolysis (%)		Log IC_{50} (mg/ml)	
		All points	Central points	All points	Central points
Pea	Average	73 ± 9	77 ± 3	-1.24 ± 0.04	-1.19 ± 0.01
	Range	49 to 86	65 to 85	- 0.90 to -1.66	-1.16 to -1.23
Whey	Average	53 ± 4	64 ± 4	-1.32 ± 0.06	-1.42 ± 0.04
	Range	32 to 82	40 to 81	-0.37 to -1.66	-1.23 to -1.66

For the pea protein, the best fitting model equation of the degree of proteolysis and log IC_{50} were respectively calculated as a linear and quadratic polynomial equation of temperature, incubation time in stomach and incubation time in small intestine phase. Table 6 shows the coefficients of the obtained response equations and the corresponding model validation parameters.

For a good model, lack of fit should be non-significant, R^2 values high (predicted R^2 in reasonable agreement with adjusted R^2), adequate precision above 4 and PRESS values low. The adequate precision value measures the signal over noise ratio, while the PRESS statistic points to the lack of predictive value of the model. Reduced models could be obtained by omitting the non-significant model terms ($p > 0.1$), taking into account the hierarchy of the model, but responses and validation parameters did not change much (data not shown). Both the linear model for the degree of proteolysis and the quadratic model for the log IC_{50} were significant. Furthermore, the model validation parameters demonstrated

that the equations were representative for the observed data and useful to navigate the design space. Figure 5 shows the response surface plots of the degree of proteolysis and the ACE inhibitory activity of the pea digest.

Table 6. Coefficients in terms of actual and coded factors and their p values, and validation parameters of the linear and quadratic model equations of respectively the degree of proteolysis (%) and the log IC_{50} value (mg/ml) of the pea protein digest as a function of temperature (°C) (A), incubation time in stomach (min) (B) and incubation time in small intestine phase (min) (C).

	Degree of proteolysis (%)			Log IC_{50} (mg/ml)		
Model	Linear (p = 0.0232)			Quadratic (p < 0.0001)		
Factors	actual	coded	p-value	actual	coded	p-value
Constant	43.441	73.2		-2.438	-1.19	
A	0.536	5.90	0.0124	0.039	-0.017	0.1494
B	0.023	1.39	0.5152	0.010	-0.096	< 0.0001
C	0.047	4.27	0.0589	$1.7 .10^{-3}$	-0.094	< 0.0001
A^2				$-1.4 .10^{-4}$	-0.017	0.1286
B^2				$-1.8 .10^{-5}$	-0.066	0.0002
C^2				$4.1 .10^{-6}$	0.033	0.0120
AB				$-1.6 .10^{-4}$	-0.100	0.0001
AC				$-7.4 .10^{-5}$	-0.073	0.0014
BC				$-1.1 .10^{-5}$	-0.057	0.0056
Lack of fit		0.65			0.20	
Adjusted R^2		0.33			0.95	
Predicted R^2		0.16			0.80	
Adequate precision		6.68			27.05	
PRESS		1442			0.08	

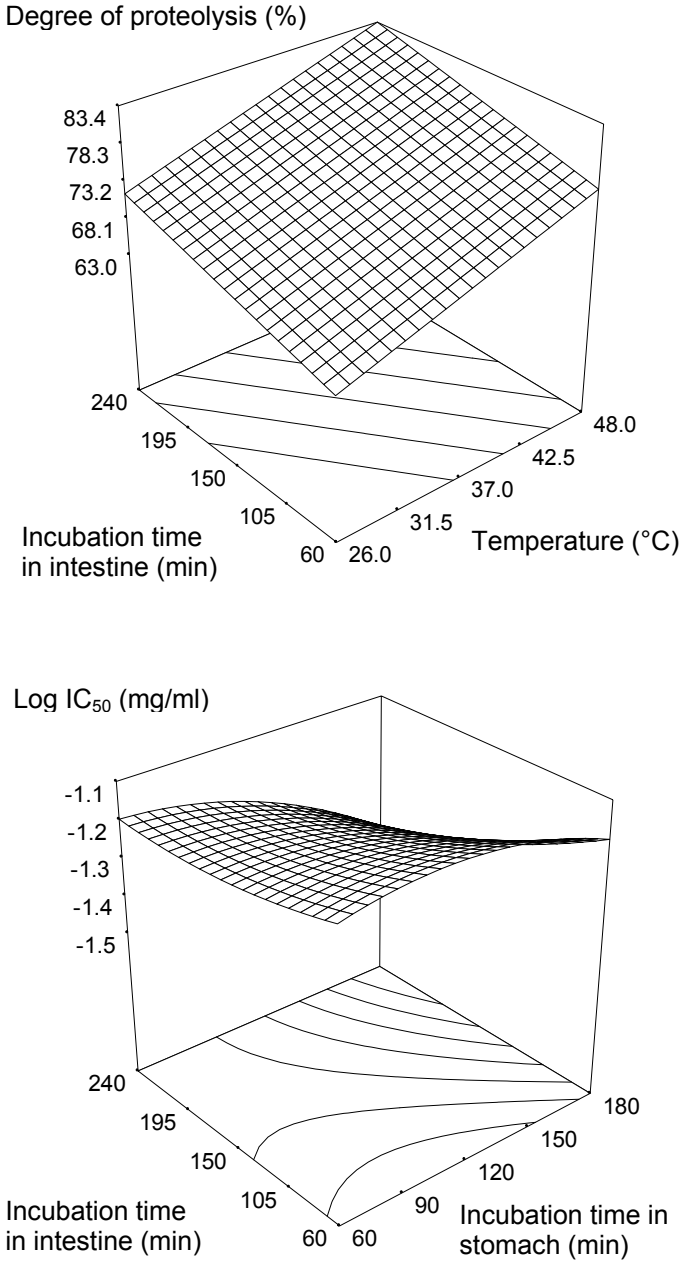

Figure 5. Response surface plots of the degree of proteolysis (%) and the log IC_{50} (mg/ml). The non-displayed parameter is set at the central point.

Results are displayed as perturbation plots of the non-reduced models and contour plots (Figure 6 and 7). A perturbation plot shows how the response changes as each factor, expressed as coded value, moves from the central point of the design towards the borders, while all other factors are held constant at the central point. The degree of proteolysis showed the major deviation from the central point when the temperature varied, while the log IC_{50} changed substantially from the central point when the incubation time in the stomach increased and the incubation time in the small intestine phase decreased.

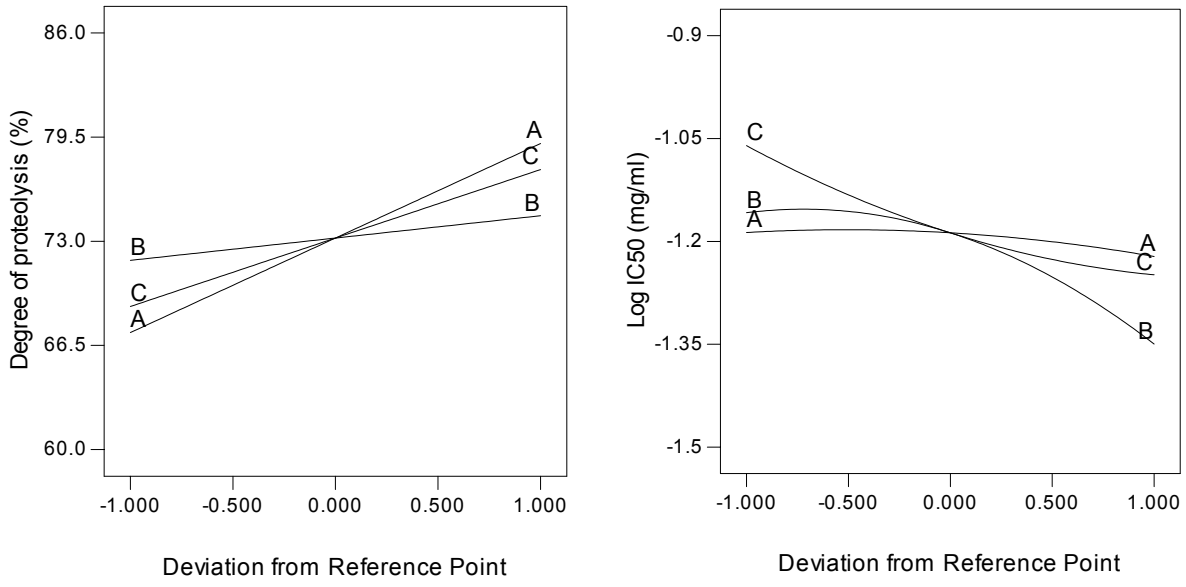

Figure 6. Perturbation plots for degree of proteolysis (%) and log IC_{50} (mg/ml) as a function of temperature (A), incubation time in stomach (B) and incubation time in small intestine phase (C).

A contour plot is a two-dimensional representation of the response for selected factors. The contour plot for the degree of proteolysis indicates that this response increased as the pea protein was digested at higher temperatures and with longer incubation times in the small intestine phase, when the incubation time in the stomach was set at 120 min. At a temperature of 37°C, the log IC_{50} lowered when both the incubation time in stomach and small intestine phase increased. In the contour plots for the log IC_{50}, where the temperature is displayed in the X axis, the rather horizontal lines point to the limited importance of the temperature.

At the central point, the observed degree of proteolysis and log IC_{50} value were respectively 77 ± 3% and -1.19 ± 0.01 mg/ml (IC_{50} = 0.065 mg/ml), while their predicted values were respectively 73% and -1.19 mg/ml. From the three-dimensional plot of the standard errors it can be found that the model for the log IC_{50} is more reliable than the one for the degree of proteolysis: the central composite design provided relatively precise predictions over a broader area around the central point (Figure 8). The circular contours confirmed the rotatability of the design.

The central composite design was used to optimise towards a maximal degree of proteolysis on one hand and a minimal log IC_{50} on the other hand, when all the parameters were in the design space. For both criteria, this resulted in maximal parameters: maximal temperature (48°C), maximal incubation time in stomach (3 h) and maximal incubation time in small intestine phase (4 h). The maximal degree of proteolysis amounted to 85% and the minimal log IC_{50} was -1.66 mg/ml (IC_{50} = 0.022 mg/ml). As this maximum was situated at the

edge of the design space, it was associated with a relatively large standard error. Hence, the optimisation results have to be interpreted with caution. Because of the non-significance of the factor incubation time in stomach in the model of the degree of proteolysis, maximal degree of proteolysis was also attained when only the temperature and the incubation time in the small intestine phase were maximised in the model. When the temperature, the least important factor in the model of the log IC_{50}, was set at 37°C, a slightly lower maximal ACE inhibitory activity (log IC_{50} = -1.47 mg/ml, IC_{50} = 0.034 mg/ml) was obtained for maximal incubation time in stomach and maximal incubation time in small intestine phase.

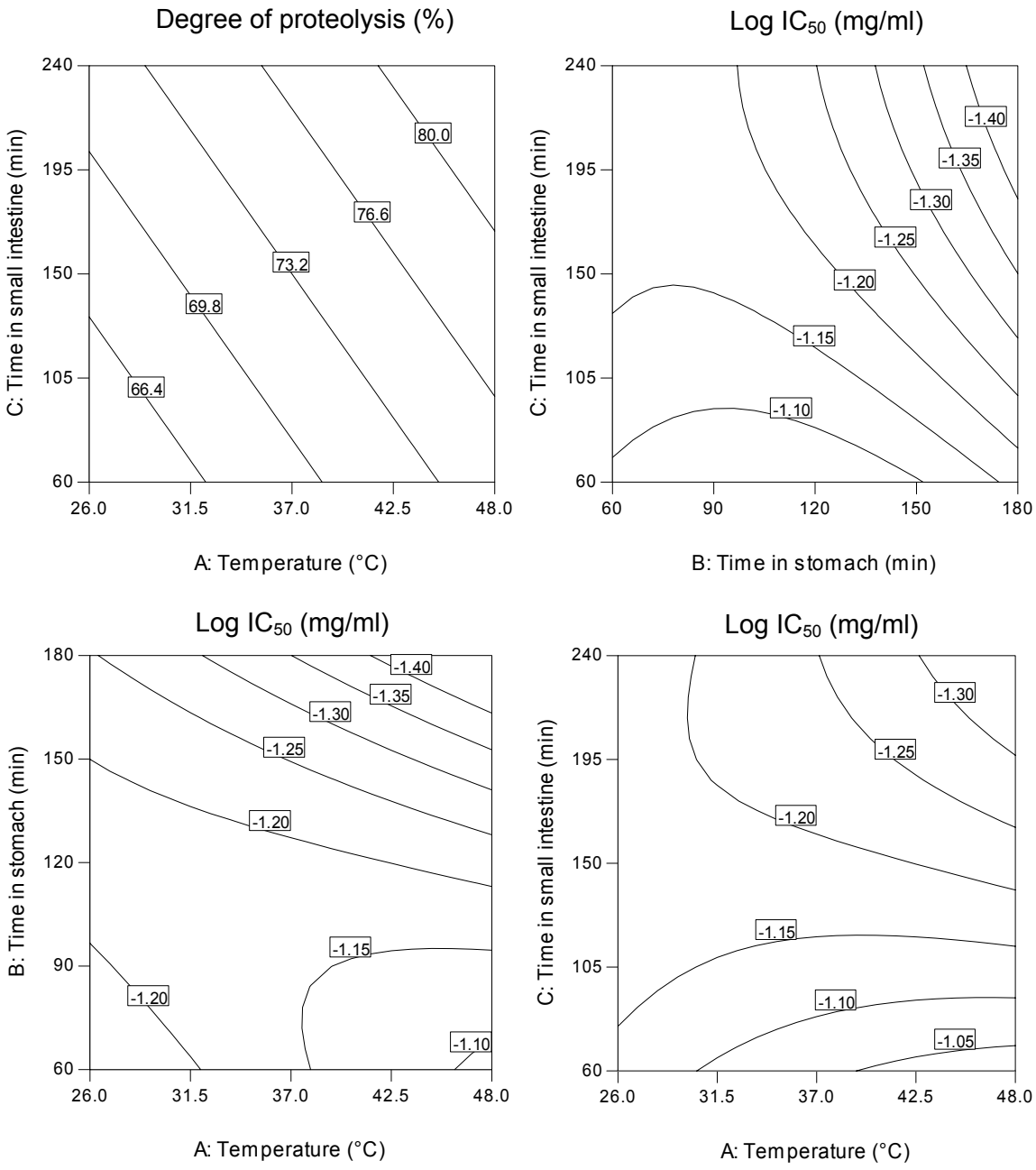

Figure 7. Two-dimensional contour plots for the degree of proteolysis (%) and log IC_{50} (mg/ml). The non-displayed parameter is set at the central point.

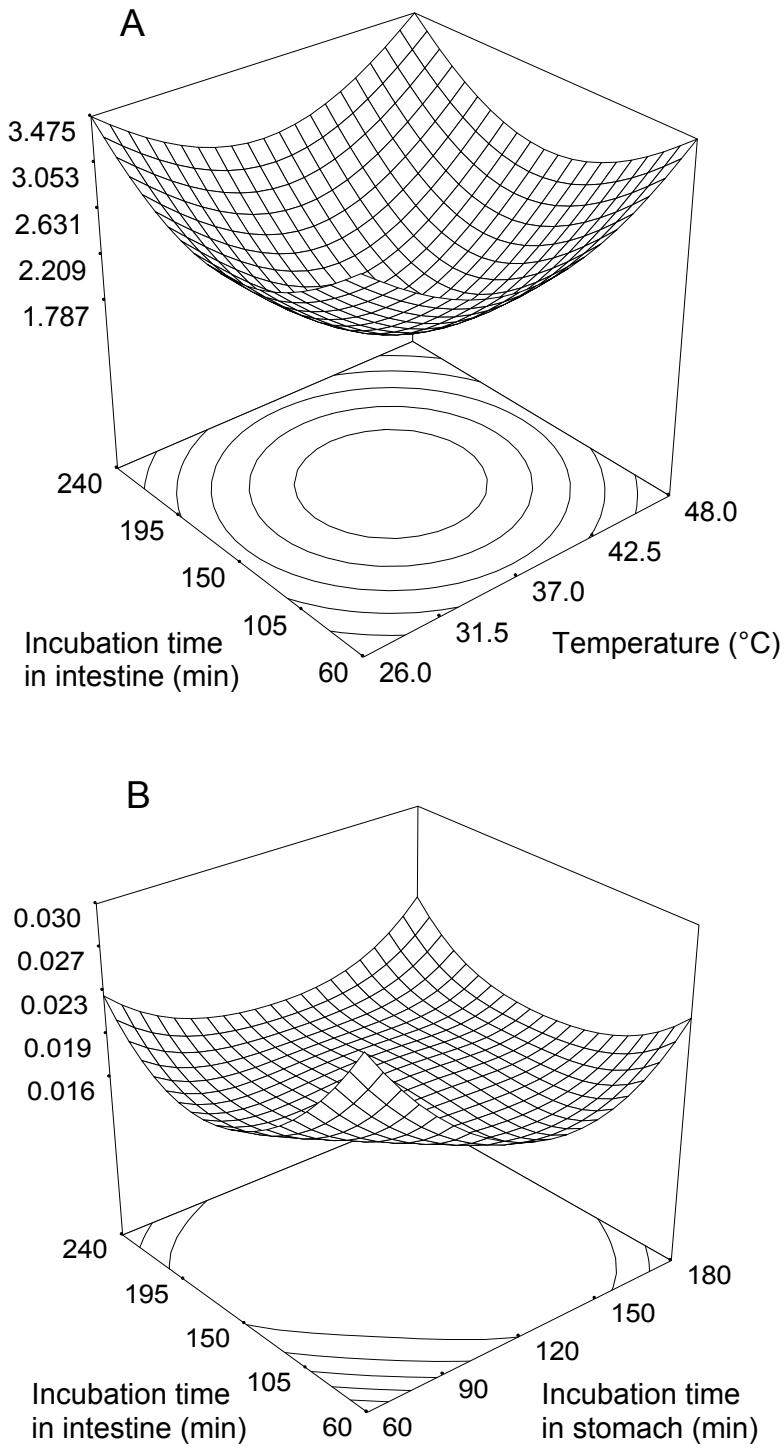

Figure 8. Three-dimensional plot of the standard error of the degree of proteolysis (%) (A) and the standard error of the log IC_{50} (mg/ml) (B) in function of the most significant parameters of the model, while the other parameter was set at the central point.

DISCUSSION

Gastrointestinal digestion is of major importance in the bioavailability of ACE inhibitory peptides. After oral administration gastrointestinal proteases may splice the ACE inhibitory peptides, thereby increasing or decreasing their activity. For example, after oral administration in spontaneously hypertensive rats (SHR) the nonapeptide Ile-Val-Gly-Arg-Pro-Arg-His-Gln-Gly, isolated from a thermolysin digest of chicken muscle, is hydrolysed by trypsin to Ile-Val-Gly-Arg-Pro-Arg, which then exerts the antihypertensive effect (Fujita *et al.*, 2000). Similarly, the ACE inhibitory activity of Lys-Val-Leu-Pro-Val-Pro-Gln, derived from β-casein, is increased by pancreatin digestion and the derivative Lys-Val-Leu-Pro-Val-Pro lowers the blood pressure of SHR (Maeno *et al.*, 1996). In the same study, an α_{s1}-casein derived peptide, Tyr-Lys-Val-Pro-Gln-Leu, with strong ACE inhibitory activity, fails to exert an antihypertensive effect due to pancreatin degradation.

Our previous study demonstrated the high ACE inhibitory activity of pea and whey protein after gastrointestinal digestion (Vermeirssen *et al.*, 2003a). Here, the process of digestion was investigated more in detail with respect to the formation of ACE inhibitory activity. During a first set of experiments, the gastrointestinal digestion was simulated in batch, while for the experimental design a semi-continuous reactor model was developed. In literature different *in vitro* models are reported: from simple batch experiments (Oomen *et al.*, 2002; Pihlanto-Leppällä *et al.*, 1998), dialysis bags (Gauthier *et al.*, 1986) to more complex computer-controlled models like artificial stomach (Yvon *et al.*, 1992), TIM (Minekus *et al.*, 1993), SHIME (Molly *et al.*, 1993) and models coupled to cell cultures (Glahn *et al.*, 2002). Although some of them use pancreatin as a small intestinal enzyme source, we preferred to use relatively pure trypsin and chymotrypsin.

Comparison of three different *in vitro* gastrointestinal digestions

Three different *in vitro* batch gastrointestinal digestions were compared to elucidate the conditions required for protein hydrolysis and release of ACE inhibitory activity. From the non-optimal over the physiological to the prolonged optimal digestion, protein hydrolysis increased significantly, due to a more optimal pH for the gastrointestinal proteases and longer incubation times. The degree of proteolysis of the pea digest levelled off from the physiological to the prolonged optimal digestion, suggesting that liberation of peptides from the pea protein reached a saturation point. Yet, the prolonged optimal digestion of the whey protein still contributed substantially to protein hydrolysis. In addition, pea protein displayed higher increases in degree of proteolysis than whey, showing that the latter is less prone to

gastrointestinal digestion. Indeed, the major whey proteins α-lactalbumin and β-lactoglobulin partially resist gastrointestinal digestion. Pepsin degrades α-lactalbumin and only denaturated β-lactoglobulin, trypsin slowly cleaves α-lactalbumin and denaturated β-lactoglobulin, while α-chymotrypsin hydrolyses both α-lactalbumin and β-lactoglobulin to a limited extent (Guo *et al.*, 1995; Schmidt and van Markwijk, 1993).

All three digests of both proteins reached 100% ACE inhibitory activity, but the 50% inhibitory concentrations differed. Both pH and incubation time had an influence on the formation of ACE inhibitory activity. A physiological digestion sufficed for both proteins to obtain minimal IC_{50} and hence maximal ACE inhibitory activity. The formation of ACE inhibitory activity reached a saturation level, after which no major breakdown occurred or equilibrium existed between the formation and degradation of ACE inhibitory peptides. Apparently, a certain degree of proteolysis was necessary to exert high ACE inhibitory activity, as the non-optimal digestion resulted in lower ACE inhibitory activity than the other two digestions and this effect was larger for whey than for pea. This may be of physiological importance since a higher, non-optimal pH in the stomach can occur in humans with increasing age (Morihara *et al.*, 2001). The higher ACE inhibitory activity of the whey protein after a physiological or prolonged optimal digestion compared to those of pea can be attributed to the amino acid sequence of the proteins which determines the presence of bioactive peptide sequences and enzyme splicing sites. The IC_{50} of the physiological and prolonged optimal whey digests are lower than the IC_{50} ranges of 0.130-0.201 and 0.345-1.733 mg/ml reported respectively by Mullally *et al.* (1997a) and Pihlanto-Leppälä *et al.* (2000) for whey protein digested by gastrointestinal proteases. However, differences in the ACE inhibition assay and the way of calculation of the ACE inhibitory activity complicate the comparison of IC_{50} values reported in literature (Ariyoshi, 1993). No IC_{50} values for pea hydrolysates are reported in literature.

Compared to the ACE inhibitory activity produced after *in vitro* gastrointestinal digestion of autoclaved pea and whey protein (Chapter 4), higher activity is obtained after digestion of the native proteins (this chapter). Denaturated proteins are usually more prone to hydrolysis (Guo *et al.*, 1995; Pihlanto-Leppälä *et al.,* 2000), although sometimes the formation of irreversible disulfide interactions may lead to polymerisation and consequently a reduced susceptibility to hydrolysis (Angelo *et al.*, 1982). No differences in change in degree of proteolysis were observed between the autoclaved and the native protein digests. An increased or decreased hydrolysis in the heat treated proteins may only be reflected at the level of soluble peptides and amino acids.

The HPLC chromatograms confirmed the difficult degradation of β-lactoglobulin in the whey digests. The higher initial peaks in the prolonged optimal pea digest present short hydrophilic peptides or amino acids. The higher similarity between the chromatograms of the different pea digests compared to the whey digests corresponds with the higher similarity observed in the IC_{50} values of the different pea digests. This can partially be explained by the higher susceptibility of pea protein for hydrolysis by gastrointestinal proteases.

Experimental design in a semi-continuous reactor model

A semi-continuous reactor model was developed with the aim to produce high ACE inhibitory activity from pea and whey protein by means of gastrointestinal proteases. As a result, the formed peptides resist the physiological digestion after oral administration (Matsui *et al.*, 2002c). The process conditions of the semi-continuous reactor differed slightly from the batch experiments in the continuous and more rigorous pH and temperature control and a slight dilution effect due to the pH control with only 1 N HCl and 1 N NaOH.

Preliminary experiments excluded the protein concentration and the enzyme over substrate ratio as parameters in the experimental design. This is in agreement with a previous study on the hydrolysis of heat-denatured whey proteins by trypsin, α-chymotrypsin, alkalase and neutrase, where different protein concentrations have no significant influence on the reaction rate (Mutilangi *et al.*, 1995). It is expected that a protein concentration of 4% or higher is sufficient to provide substrate saturation conditions. In the same work, increasing enzyme over substrate ratios augment the degree of proteolysis. This may explain the slight, but non-significant decrease in IC_{50} for increasing enzyme over substrate ratios. For fish protein hydrolysates, increase in enzyme concentration and/or hydrolysis time is shown to result in improved ACE inhibitory activity (Matsui *et al.*, 1993). A similar evolution is obtained during the digestion of blood plasma albumin by alkalase, while during digestion of casein by trypsin the high ACE inhibitory activity obtained the first hour, decreases temporarily and then increases gradually with hydrolysis time and increasing enzyme concentration (Hyun and Shin, 2000). Since the optimal temperatures of the different gastrointestinal enzymes were slightly higher than the physiological temperature of 37°C (Angelo *et al.*, 1982; Ryle, 1984; Venkatesk and Sundaram, 1998), the effect of this parameter was investigated in the design. The batch physiological digestion sufficed to achieve maximal ACE inhibitory activity and therefore served as a central point in the experimental design. Consequently, the digestion was performed at physiological pH, which is close to the optimal pH for the gastrointestinal proteases (Ganapathy and Leibach, 1999).

For the whey protein, high experimental error prevented the design of a significant model for both responses. The higher variability of the whey protein in comparison to the pea protein is also observed in higher standard errors for the degree of proteolysis and the log IC_{50} in the comparison of the three batch digestions. This can partially be explained by the lower susceptibility to hydrolysis, resulting in higher variations when slightly different conditions are applied.

By means of the central composite design for pea, a linear model for the degree of proteolysis was established, where the incubation time in the stomach phase was of non-significant importance. Gauthier *et al.* (1986) found that the degree of proteolysis of a casein hydrolysate increases with longer incubation times in the presence of pancreatin, while a short or long pre-incubation with pepsin has the same effect. Higher temperature exerted a major effect, which can be attributed to the optimal temperatures of the enzymes, which are above 37°C (Angelo *et al.*, 1982; Ryle, 1984; Venkatesk and Sundaram, 1998). For the ACE inhibitory activity, expressed as log IC_{50}, a quadratic equation described the relationship between temperature, incubation time in stomach and incubation time in small intestine phase. Here, the temperature and its quadratic term exerted no significant influence, but it could not be omitted in the model because of the hierarchy and the significant interaction terms. As seen from the contour plots longer incubation times in the stomach yielded higher ACE inhibitory activity and this effect was more pronounced for a long incubation time in the small intestine. This is somewhat contradictory to the results obtained for the batch prolonged optimal and physiological digestion for pea, but there the lack of pH control may have resulted in lower ACE inhibitory activity for longer incubation times. The fact that two different models were obtained for the two responses indicates that no straight forward relationship exists between the (log) IC_{50} and the degree of proteolysis during digestion, which was also observed for the comparison of the three batch digestions and was already reported in literature (Mullally *et al.*, 1997a).

During the redaction of this paper, one very recent study appeared on the optimisation of ACE inhibition by response surface methodology (van der Ven *et al.*, 2002). In this paper, a quadratic model for the IC_{50} of a pancreatic whey digest is obtained. Whereas we studied both the stomach and small intestine phase of the gastrointestinal digestion, this study focusses only on the small intestine phase as a production process for ACE inhibitory peptides. Moreover, the authors included pH, enzyme over substrate ratio and pre-treatment temperature of the protein as process parameters, in addition to temperature and hydrolysis time. They found maximal ACE inhibitory activity at rather low pre-treatment temperature of the protein and for hydrolysis at high enzyme over substrate ratio, long hydrolysis time

(optimally 5 h), pH 8-9 and a temperature of 45°C. Hence, their findings on hydrolysis time and temperature are in agreement with our results for the pea digest.

In conclusion, gastrointestinal digestion is physiologically important after oral administration of ACE inhibitory peptides. The normal physiological process resulted in maximal ACE inhibitory activity release from pea and whey protein. When performed in a semi-continuous reactor model, it could be used as a production process for ACE inhibitory peptides from pea and whey protein. For pea, a response surface model indicated the process parameters necessary to release maximal ACE inhibitory activity.

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CHAPTER 6

ACE INHIBITORY *IN VITRO* GASTROINTESTINAL DIGESTS: TIME COURSE STUDY AND PROTEIN DEGRADATION

Redrafting after VERMEIRSSEN, V., VAN DER BENT, A., VAN CAMP, J. and VERSTRAETE, W. Release of angiotensin I converting enzyme (ACE) inhibitory activity during *in vitro* gastrointestinal digestion: time course study and characterisation of protein degradation. *In preparation.*

Chapter 6

ACE inhibitory in vitro gastrointestinal digests: time course study and protein degradation

ABSTRACT

In vitro gastrointestinal digestion simulating the *in vivo* conditions of protein digestion produced high ACE inhibitory activity from pea and whey protein. A 2.73 mg/ml pea digest showed maximal ACE inhibitory activity already in the early stomach phase and its IC_{50} value further decreased in the small intestine phase. For a 2.73 mg/ml whey digest, however, the level of 100% ACE inhibitory activity was only attained in the small intestine phase. Subsequent supplementation of a rat intestinal acetone powder, which simulated the digestion by brush border enzymes, resulted in an increase in IC_{50} value for both proteins, and more for whey than pea. Nevertheless, a substantial amount of peptides were still ACE inhibitory active after digestion by gastrointestinal and brush border enzymes and the final IC_{50} values were 0.093 mg/ml for pea and 0.128 mg/ml for whey. SDS-PAGE showed that the major part of the pea proteins was already broken down in the stomach phase. The major whey protein β-lactoglobulin was only degraded from the small intestine phase onwards. The presence of known ACE inhibitory peptides in the pea and whey protein sequences was studied using a dedicated computer program. This program makes use of a database of about 500 reported ACE inhibitory sequences and their IC_{50} values, and produces a theoretical score, weighted on the total number of amino acids, for the potential of ACE inhibitory peptides in a given source protein. For pea, vicilin (VCLC_PEA) and albumin PA2 (PA2_PEA) obtained scores as high as 10 on ACE inhibitory activity, compared to 16 for β-casein (CASB_BOVIN) taken as a reference protein. β-lactoglobulin (LACB_BOVIN) exceeded all other proteins with a score of 26. *In silico* digestion of these proteins by pepsin, trypsin and α-chymotrypsin directly released two ACE inhibitory peptides from PA2, one from vicilin and one from β-lactoglobulin with an IC_{50} lower than 100 µM. Several less active ACE inhibitory peptides and precursor peptides, which may form ACE inhibitory peptides upon further digestion by brush border, enterocyte or plasma peptidases, were also released.

Keywords: ACE inhibitory peptides, brush border, SDS-PAGE, pea protein, whey protein, ACE inhibitory peptide database

INTRODUCTION

Foods containing angiotensin I converting enzyme (ACE, EC 3.4.15.1) inhibitory peptides have shown to be effective in both the prevention and treatment of hypertension (Sipola *et al.*, 2002; Takano, 1998). As hypertension indirectly contributes to cardiovascular mortality in the developed world, food derived ACE inhibitors may provide an alternative or an additional cure to antihypertensive drugs (Fitzgerald and Meisel, 2000). Besides, ACE inhibitory peptides have not shown any side effects yet, which makes them attractive in the prevention of a high blood pressure as well.

Many ACE inhibitory peptides have been characterised from both vegetable and animal proteins (Yamamoto, 1997). To exert an antihypertensive effect *in vivo*, these bioactive peptides have to reach the blood stream in an active form upon oral administration (Masuda *et al.*, 1996). Gastrointestinal proteases may metabolise the ingested ACE inhibitory peptides, resulting in either the release of more active peptides or inactive fragments (Maeno *et al.*, 1996). It is generally accepted that potent ACE inhibitory peptides exhibit partial resistance to complete degradation by human peptidases (Meisel, 1998). In addition, ACE inhibitory peptides have to cross the intestinal enterocytes to reach the blood stream. In this respect, di- and tripeptides are most easily absorbed (Webb, 1990).

Although their precise structure-activity relationship is not fully understood, competent ACE inhibitory peptides contain hydrophobic (aromatic or branched side-chains) amino acid residues at the three C-terminal positions. However, a C-terminal lysine or arginine, with a positive charge on the ε-amino group, also seems to contribute substantially to the inhibitory potency (Fitzgerald and Meisel, 2000). The amino acid sequence of a given protein not only determines the potential of ACE inhibitory peptides inactively present, but also their specific release by enzyme cleavage. Therefore, it is possible to predict the biological activity of protein fragments using sequence alignments between proteins and known ACE inhibitory peptides collected in a database (Dziuba *et al.*, 1999b). Such a database can be combined with information on recognition sequences of proteolytic enzymes, which are available on the World Wide Web (e.g. PeptideCutter at http://www.expasy.org), in order to estimate the specific release of bioactive peptides.

Pea protein isolate contains the globulin proteins legumin, vicilin and convicilin and the albumin fraction that comprises proteins with a biological function in the pea seed. The major representatives of the latter fraction are PA1 and PA2 albumin (Guéguen, 2000). Whey protein isolate contains in order of importance β-lactoglobulin, α-lactalbumin, immunoglobulins, BSA and some minor proteins (Wong *et al.*, 1996) (Chapter 1).

In previous studies, high ACE inhibitory activity has been produced by different *in vitro* gastrointestinal digestions of pea and whey protein (Vermeirssen *et al.*, 2003a; Vermeirssen *et al.*, 2003b). An *in vitro* digestion simulating the physiological conditions *in vivo* is adequate for the formation of maximal ACE inhibitory activity. Moreover, whey hydrolysates are more ACE inhibitory potent than pea hydrolysates (Vermeirssen *et al.*, 2003b). In this paper, this physiological digestion was studied over time and followed by a rat intestinal acetone powder digestion to observe the evolution of the ACE inhibitory activity in the different stages of gastrointestinal digestion. Hence, the formation and stability of the ACE inhibitory peptides released from pea and whey protein up to the brush border barrier were investigated. Moreover, the degradation of the different proteins in pea and whey protein was followed by means of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). These experimental data were combined with the information obtained from a database for ACE inhibitory peptides, a protein database and a protein cleavage program. In this way, the proteins in pea and whey protein containing potent ACE inhibitory peptides could be distinguished and the theoretical release of these bioactive peptides by gastrointestinal proteases was studied.

MATERIALS AND METHODS

Products

The products used during *in vitro* gastrointestinal digestion, HPLC analysis and the ACE inhibition assay are described in Chapter 5. Rat intestinal acetone powder (I 1630) was purchased from Sigma-Aldrich (St.-Louis, MO, USA). Non-specified products were analytical grade from VWR International (Zaventem, Belgium).

In vitro gastrointestinal digestion followed by brush border phase

The experimental conditions of the *in vitro* physiological digestion are mentioned in Chapter 5. A 150 ml 4% (w/v) protein solution was used and the brush border phase was simulated by addition of rat intestinal acetone powder in an E/S of 1/10 (w/w) and incubation for 1 h at 37°C. This rat intestinal acetone powder contains all enzymes from rat intestinal tissue that do not phase separate into acetone. Samples were taken at the start and after 0.5 h, 1 h, 2 h, 2.5 h, 3.5 h, 4.5 h, 5 h and 5.5 h incubation.

Degree of proteolysis and ACE inhibitory activity

For the treatment of the samples and the analyses, reference is made to Chapter 4. The measurement of the ACE activity and ACE inhibitory activity in 4 mg/ml rat intestinal acetone powder solution was done as described in Chapter 2.

Another way to indicate a degree of proteolysis is by analysing the number of free amino groups as a measure of the number of peptide bonds cleaved. The TNBS method, where trinitrobenzenesulfonic acid reacts with primary amines to form a chromophore, was applied in this case (Adler-Nissen, 1979; Panasiuk *et al.*, 1998).

SDS-PAGE

Samples were diluted five times in 10 mM Tris buffer, pH 8, containing 2.5% (w/v) SDS, 5% (w/v) β-mercapto-ethanol and 1 mM EDTA and boiled for 5 min. The low molecular weight (LMW) electrophoresis calibration kit (Amersham Pharmacia Biotech, Uppsala, Sweden) was used as standard: phosporylase b (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). The standard was boiled for 1 min. Gel electrophoresis was performed with a Phastsystem (Pharmacia). Phastgel SDS buffer strips, containing 0.55% (w/v) SDS in 0.2 M tricine and 0.2 M Tris buffer at pH 8.1, were used. One microlitre diluted sample or standard was spotted on Phastgel Homogeneous 20 gels to analyse the protein/peptide fraction between approximately 1 and 100 kDa. Electrophoretic conditions programmed into the Phastsystem were 15°C, 250 V, 3 W and 10 mA for 1 Vh, subsequently 1 mA for 1 Vh and finally 10 mA for 95 Vh. For the detection of the protein bands in the gel, Coomassie blue staining was applied. The gels were scanned and analysed by means of Image Master TotalLab v1.00 (Pharmacia).

HPLC

The HPLC procedure for the samples taken at different times during the digestion of pea protein and of pea and whey protein subjected to only a stomach or small intestine phase digestion is equal to the one mentioned in Chapter 5. A control solution containing 16 mg of pepsin, trypsin and α-chymotrypsin and 400 mg rat intestinal acetone powder in 100 ml MilliQ, centrifuged for 15 min at 10 000 g was also eluted by the HPLC programs for pea and whey, respectively (Chapter 5).

In silico screening of the ACE inhibitory peptide potential

A theoretical analysis of ACE inhibitory peptides present in the sequences of pea and whey proteins was performed using a database and data-mining software developed by the Institute for Agronomical and Technological Research (ATO) (Wageningen, The Netherlands). The database contains 498 ACE inhibitory peptides published in literature. The data-mining software screens this database against a second database in SwissProt format, containing the sequences of source proteins of interest, in this case from pea and whey. For more than 95% of these peptides an IC_{50} value is also available. The inclusion of the latter enables the program to add up the total activity of the peptides identified in a certain protein sequence, after which the summed score is 'weighted' to compensate for the relative size of the examined protein (i.e. the total number of amino acids). ACE inhibitory peptide scores are attributed to the proteins in two ways. When scoring on high ACE inhibitory activity, the peptides in the database are ranked from high to low ACE inhibitory activity and in this order the program searches for homologous peptide sequences in the food protein. When scoring on peptide size, the database is first sorted on the number of amino acids within a specific peptide and then on ACE inhibitory activity. Subsequently, sequence alignments between the food protein and the database are discovered. When ACE inhibitory peptide sequences overlap, depending on the scoring method, the peptide with the highest activity or the shortest peptide with the highest activity is chosen.

Protein sequences from pea and whey protein were found on the World Wide Web in the protein databases SwissProt and TrEMBL at the ExPASy Molecular Biology Server (http://www.expasy.org/sprot) and in the National Center for Biotechnology (NCBI) protein database (http://www.ncbi.nlm.nih.gov). Complete sequences were preferred over fragments, because most sequence fragments are not representative for actual mature proteins. Signal peptides and propeptide sequences were removed from precursor protein sequences from the SwissProt and TrEMBL database. Several proteins were used as entry in the ACE inhibitory peptide database search (Table 1). Caseins were used as reference proteins as already many ACE inhibitory peptides are derived from these food proteins (Fitzgerald and Meisel, 2000). For the proteose peptone C5/8 fraction (Kinsella and Whitehead, 1989) and casein glycomacropeptide (CMP) fragments of proteins were used in the screening. The protein monomers of the pea albumin PA1 fraction were separated by a propeptide in the precursor protein sequence ALB1_PEA in the SwissProt database.

Table 1. Proteins from pea and whey with their entry names in the protein databases.

Protein	Entry name	Protein database
PEA		
vicilin	VCLC_PEA	SwissProt
legumin J	LEGJ_PEA	SwissProt
legumin A2	LEG2_PEA	SwissProt
legumin A	LEGA_PEA	SwissProt
legumin B	O24294	TrEMBL
legumin K	S26688	NCBI
albumin PA2	ALB2_PEA	SwissProt
vicilin 47 kDa	Q43626	TrEMBL
vicilin 14 kDa	VCL1_PEA	SwissProt
convicilin	Q9M3X6	TrEMBL
convicilin A	CVCA_PEA	SwissProt
albumin PA1B (leginsulin)	ALB1_PEA [part2]	SwissProt
albumin PA1A	ALB1_PEA [part1]	SwissProt
WHEY		
β-lactoglobulin	LACB_BOVIN	SwissProt
β_2-microglobulin	B2MG_BOVIN	SwissProt
serotransferrin	TRFE_BOVIN	SwissProt
lactotransferrin	TRFL_BOVIN	SwissProt
bovine serum albumin (BSA)	ALBU_BOVIN	SwissProt
proteose peptone C5/C8	CASB_BOVIN [f(1-107)]	SwissProt
lactoperoxidase	PERL_BOVIN	SwissProt
α-lactalbumin	LCA_BOVIN	SwissProt
lysozyme	LYCN_BOVIN	SwissProt
$IgG_1/_2$ heavy chain precursor	S22080	NCBI
proteose peptone C3	LCTN_BOVIN	SwissProt
casein glycomacropeptide (CMP)	CASK_BOVIN [f(106-169)]	SwissProt
CASEIN		
β-casein	CASB_BOVIN	SwissProt
α_{s2}-casein	CAS2_BOVIN	SwissProt
α_{s1}-casein	CAS1_BOVIN	SwissProt
κ-casein	CASK_BOVIN	SwissProt

In silico gastrointestinal digestion

Some protein sequences were cleaved by the combination of pepsin, trypsin and α-chymotrypsin with the software PeptideCutter at http://www.expasy.org. In parallel, the protein was cleaved by trypsin and α-chymotrypsin respectively, for which specific cleavage probabilities could be reproduced. In our figures a cleavage probability less than 60% for these enzymes is indicated by a dotted arrow.

The hydrolysis preference of the proteases in the program was the following:

- pepsin high specificity (pH 1.3): C-terminus of Phe and Leu
- pepsin low specificity (pH ≥ 2): N- and C-terminus of Phe, Tyr, Trp and Leu
- trypsin: C-terminus of Arg and Lys
- α-chymotrypsin high specificity: C-terminus of Trp, Tyr and Phe
- α-chymotrypsin low specificity: C-terminus of Trp, Tyr, Phe, Leu, Met and His

The release of ACE inhibitory sequences from the proteins by these gastrointestinal enzymes was investigated.

Statistical analysis

All values are reported as mean ± standard error (SE) of the mean (n_{min} = 3). The degree of proteolysis, ACE inhibitory activity and log IC_{50} of the pea and whey protein were compared by an independent Student t-test after the stomach, small intestine and brush border phase. For the log IC_{50}, a General Linear Model Univariate Analysis of Variance procedure (SPSS 11.0.1, Chicago, Illinois, USA) was carried out with type of protein (p_{prot}) and the phase of digestion (p_{phase}) as factors. When there was a significant type of protein × phase of digestion interaction, for both proteins a Oneway ANOVA analysis was carried out by phase of digestion. All data used in the variance analysis met the homogeneity of variance requirement assessed by Levene's test. Indication of subgroups in phase of digestion was done by Tukey post hoc test ($p < 0.05$).

RESULTS

Formation of ACE inhibitory activity over time

The physiological digestion that simulated the *in vivo* conditions of protein digestion was studied over time. To investigate the influence of brush border peptidases on the ACE inhibitory activity, the digests were subsequently incubated in the presence of rat intestinal acetone powder. The evolution of the degree of proteolysis in comparison to the percent ACE inhibitory activity of a 2.73 mg/ml sample is plotted in Figure 1, together with the log IC_{50} value after the different phases in digestion. The IC_{50} values can be found in Table 2.

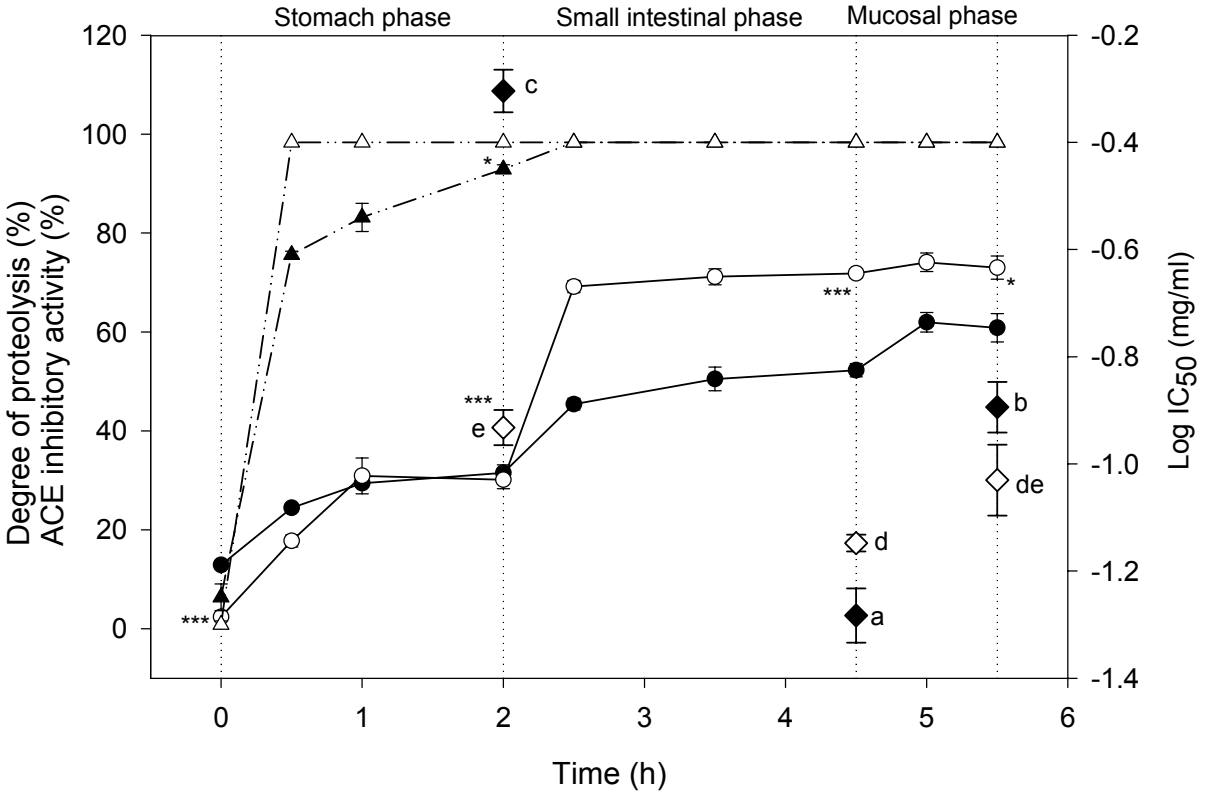

Figure 1. Evolution in the degree of proteolysis (%) (●) and ACE inhibitory activity (%) (▲) during the physiological digestion of pea (white) and whey (black) protein followed by a rat intestinal acetone powder digestion, and the log IC_{50} (♦) after the stomach, small intestine and brush border phase (n_{min} = 3). An independent samples t-test indicated the significant difference between both proteins (*p < 0.05, ***p < 0.001) at the start and after stomach, small intestine and brush border phase. Different letters separate significantly different (p < 0.05) digestion phases in log IC_{50}.

During digestion with pepsin, the degree of proteolysis climbed the first hour of digestion to 30% for both proteins and remained at that level the next hour. After the addition of trypsin

and α-chymotrypsin, the degree of proteolysis increased sharply the first half hour and thereafter only marginally to a value of 72% for pea and 52% for whey at the end of the small intestine phase ($p < 0.001$). The degree of proteolysis augmented more for pea than whey in both the stomach and small intestine phase. The peptidases from the rat intestinal extract caused only a small increase in degree of proteolysis for pea, while for whey a substantial augmentation was obtained the first half hour. Although in the mucosal phase no spectacular increases in degree of proteolysis were observed, the TNBS method indicated here major releases of free α-amino-groups for both proteins (data not shown). Final degrees of proteolysis were 73 ± 2% for the pea and 61 ± 3% for the whey digest ($p = 0.017$).

The ACE inhibitory activity presented a different evolution during digestion. For the pea protein, maximal ACE inhibitory activity (100%) at a concentration of 2.73 mg/ml was reached early in the stomach phase and this level was maintained during the small intestine and brush border phase. For the whey protein on the other hand, the ACE inhibitory activity reached 79% after half an hour of pepsin digestion and 95% at the end of the stomach phase ($p = 0.029$). Maximal ACE inhibitory activity of the whey protein was only obtained in the small intestine phase and then maintained for the remainder of the digestion. The IC_{50} values gave more information of the evolution of the ACE inhibitory activity. The IC_{50} value of whey changed drastically over the different digestion phases ($p_{phase} < 0.001$), while for pea slight variations occurred ($p_{phase} = 0.025$) with a significant difference between the ACE inhibitory activity of the stomach and small intestine phase ($p_{interaction} < 0.001$). At the end of the stomach phase, a relatively high IC_{50} value of 0.496 mg/ml was found for whey compared to 0.117 mg/ml for pea ($p < 0.001$). The IC_{50} decreased to 0.052 mg/ml for whey and 0.071 mg/ml for pea ($p = 0.063$) during the small intestine phase. In the mucosal phase, the IC_{50} values increased again, more for whey than pea, to final values of 0.093 mg/ml for pea and 0.128 mg/ml for whey ($p = 0.18$).

The angiotensin I converting enzyme is also present in the brush border, where it contributes to the digestion of oligopeptides (Yoshioka *et al.*, 1987). To account for a possible under- or overestimation of the ACE inhibitory activity in the mucosal digests, the ACE activity and the ACE inhibitory activity of 4 mg/ml rat intestinal acetone powder, the concentration used in the digestion, were studied. The rat intestinal acetone powder showed an ACE activity of 7 ± 1 U/l, compared to the ACE enzyme in the assay which was 28 U/l, and an ACE inhibitory activity below the detection limit of the assay. Due to a possible presence of ACE in the inhibitory samples, an underestimation of the ACE inhibitory activity may have occurred in the ACE inhibition assay. As the digests were further manipulated by centrifugation and lyophilisation, the maximal underestimation of the ACE inhibitory activity in the brush border phase samples was 25%.

Table 2. IC_{50} (mg/ml) of the non-digested proteins and after the stomach, small intestine and brush border phase of the *in vitro* physiological digestion of pea and whey protein.

Digestion phase	Pea	Whey
None	16	18
Stomach	0.117	0.496
Small intestine	0.071	0.052
Brush border	0.093	0.128

Follow-up of the protein degradation over time

The degradation of the pea and whey protein over time was studied by means of SDS-PAGE (Figure 2) (LMW = Low Molecular Weight Standard and C = control containing 16 mg pepsin, trypsin, chymotrypsin and 400 mg rat intestinal acetone powder in 100 ml, the concentrations present in the digests). Pepsin alone and in combination with trypsin and chymotrypsin gave no visual bands (data not shown).

The SDS-PAGE of the pea protein at the start presented the proteins released from the albumin and globulin fraction by β–mercapto-ethanol (Guéguen, 2000). At the start, about 14 bands were visible with average molecular weights of approximately 96 and 72, 46, 38, 33, 30, 28, 26, 22, 17, 15, 14 and approximately 11 and 8 kDa. Based on literature, these bands could be assigned to the major pea proteins (Figure 2). The band of 96 kDa is probably lipoxygenase, which has a reported molecular weight of 91 kDa (Crevieu *et al.*, 1997). Convicilin appears as one major band at 70-72 kDa, the molecular weight of its monomer (Croy *et al.*, 1980). The characteristic bands of vicilin are around 46, 33, 30, 20, 17 and 14 kDa (Crevieu *et al.*, 1997; Guéguen *et al.*, 1984; Nielsen *et al.*, 1988). The acidic and basic monomers of legumin correspond with bands around 40 and 20 kDa, respectively (Guéguen *et al.*, 1984). The monomer of the albumin PA2 fraction has a reported molecular weight of 26-28 kDa (Crevieu *et al.*, 1997; Croy *et al.*, 1984). The albumin PA1 fraction consists of 2 monomers of 6 and 4 kDa respectively (Guéguen, 2000). After 2 h digestion in the stomach phase, 7 major bands and on some gels (between parentheses) 3 additional bands with average molecular weights of 72, (48), (38), 28, 20, 16, 15 and approximately 13, 11 (overlapping) and (10) kDa were observed. Although already a substantial degradation of the proteins by pepsin took place, convicilin, PA2 and to a lesser extent vicilin and legumin resisted this hydrolysis. The proteins were almost completely broken down after the small intestine phase and the most intense bands were overlapping below 14 kDa, with sometimes bands at (30), (25) and (18) kDa. Addition of brush border peptidases did not change this band profile much: (25), (18) kDa and overlapping bands below 14 kDa.

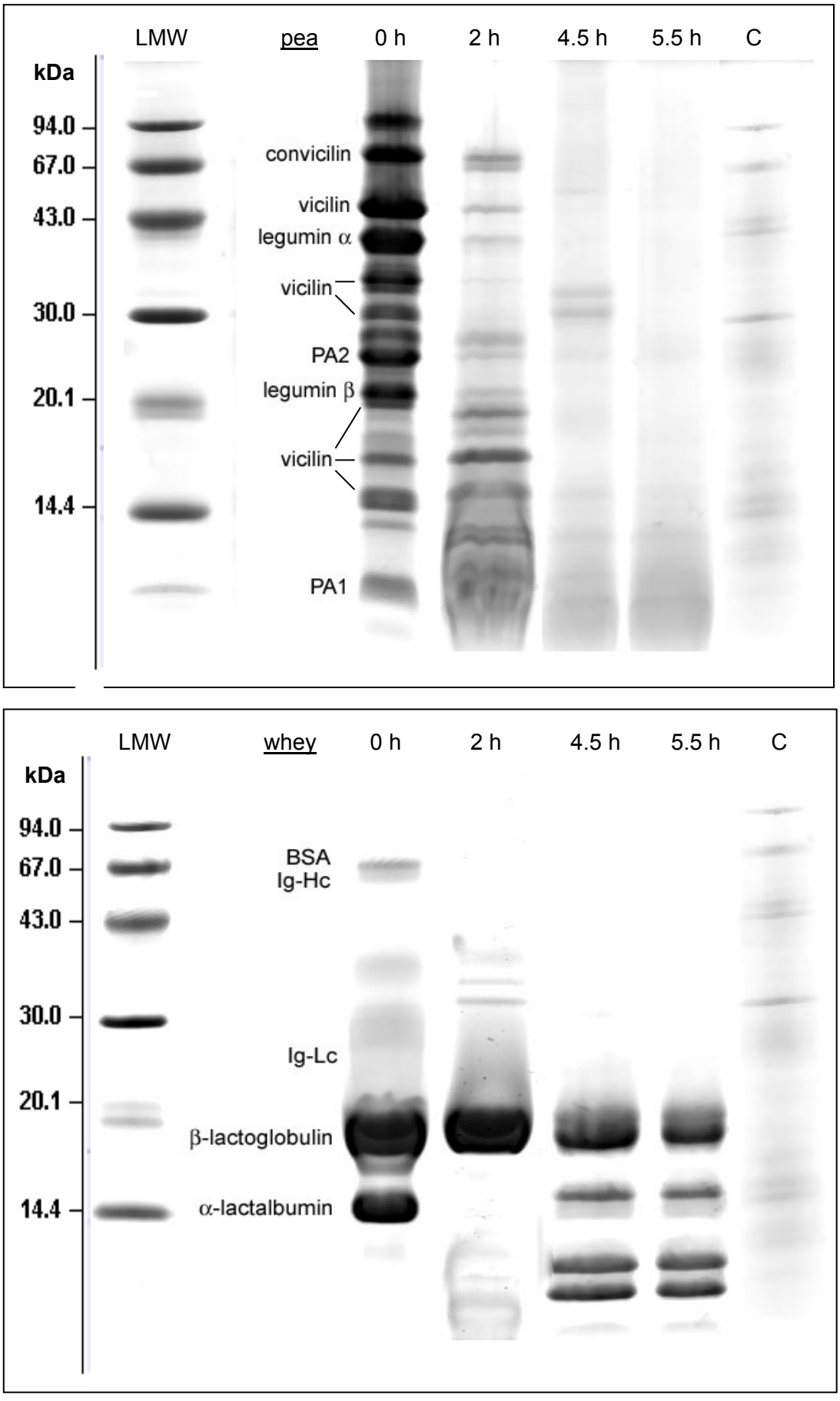

Figure 2. SDS-PAGE of pea and whey during *in vitro* physiological digestion (n_{min} = 4).

Before digestion of whey, bands with average molecular weights of (82) (secretory component associated with IgA), 64 (bovine serum albumin and heavy chain (Hc) of the immunoglobulins), 35, 29 (light chain (Lc) of the immunoglobulins), 18 (β-lactoglobulin), 14 (α-lactalbumin) and approximately (12) (proteose peptone fraction) kDa were observed (Figure 2) (Kilara and Harwalkar, 1996). After the stomach digestion the bands of 35 and 18 remained clearly present, while a fraction below 14 kDa appeared. Hence, β-lactoglobulin resisted pepsin digestion, while the other whey proteins were almost completely degraded. After the small intestine phase, bands with average molecular weights of 17, 15 and approximately 12 and 11 kDa were clearly visible in addition to smaller bands at 33 and approximately 8 kDa. Apparently, β-lactoglobulin was spliced by the action of trypsin and α-chymotrypsin into 3 smaller proteins. Finally, upon the addition of the rat intestinal acetone extract a similar profile was observed with 4 major bands at 18, 15 and approximately 12 and 11 kDa and a smaller one at approximately 8 kDa.

The HPLC profiles of the pea and whey digests after the stomach, small intestine and brush border phase were also studied. They were compared with the start profiles found in Chapter 5. A remarkable change in the peak profile from the stomach to the small intestine phase was observed. The intensity of the peaks that eluted first, increased, while the intensity of the peaks that eluted at the end of the gradient, decreased. This is due to the action of trypsin and α-chymotrypsin that degrade the proteins to smaller oligopeptides. The degradation of β-lactoglobulin mainly took place during the small intestine phase, which is in accordance with the SDS-PAGE profiles. After the stomach phase, 94% of β-lactoglobulin could be recovered, after the small intestine phase this was 51% and after the brush border digestion still 50% of β-lactoglobulin was present. The higher degradation of β-lactoglobulin during these digestion experiments compared to the ones in Chapter 5, may be explained by small differences in experimental conditions. When the lactokinin Ala-Leu-Pro-Met-His-Ile-Arg was added to a physiological digest sample and eluted by HPLC, its retention time was 14.1 min, which was about the same as in the standard curve (13.8-14.1) (Chapter 3). In many, although not in all, physiological digests, a small peak at this retention time could be observed. Therefore, the lactokinin may be released during the physiological digestion, although in small quantities.

In silico screening of the ACE inhibitory peptide potential and release

Complete amino acid sequences of proteins were screened for ACE inhibitory peptides by means of the ACE inhibitory peptide database and data-mining software developed by ATO

(Wageningen, The Netherlands). The proteins were scored by the total ACE inhibitory activity and by the presence of short ACE inhibitory peptide sequences (Table 3).

For the pea proteins, vicilin (VCLC_PEA) scored the best on shortest ACE inhibitory peptide sequences and its score for total ACE inhibitory activity was slightly higher. Hence, vicilin contained several potent ACE inhibitory peptides. The albumin PA2 (ALB2_PEA), however, had the highest score for total ACE inhibitory activity, while its score on ACE inhibitory peptide size was much lower. This signifies that this protein consisted of longer ACE inhibitory peptides that contained smaller peptides with lower ACE inhibitory activity. For albumin PA1 (ALB1_PEA), the pea protein that scored the worst, equal scores for ACE inhibitory activity and peptide size were obtained. This means that there were no peptides present for which there is a longer overlapping equivalent available or that there was a longer equivalent in the protein sequence present, but that it is less active than one of its shorter constituents. The highest number of ACE inhibitory peptide sequences was present in convicilin (Q9M3X6), the longest pea protein sequence. The different genetic variants of respectively legumin and convicilin displayed similar scores.

The different whey proteins exhibited larger differences in scores compared to pea. β-lactoglobulin (LACB_BOVIN) had the highest scores on total ACE inhibitory activity and shortest ACE inhibitory peptide sequence, not only of the whey proteins, but of all the proteins screened by the database. The iron-binding transferrins (TRFE_BOVIN, TRFL_BOVIN) scored high on total ACE inhibitory activity, but their scores on shortest peptide sequence were considerably lower. Apparently these proteins contained potent ACE inhibitory peptide sequences that included smaller peptides with lower activity. The other whey proteins screened had scores that were comparable with pea. The amino acid sequences of α-lactalbumin (LCA_BOVIN) and lysozyme (LYCN_BOVIN) are alike (Wong *et al.*, 1996) and therefore both proteins succeeded each other in the ACE inhibitory peptide scoring.

The caseins were also analysed by the ACE inhibitory peptide database, as casein-derived ACE inhibitory peptides have been well characterised (Fitzgerald and Meisel, 2000; Pihlanto-Leppälä, 2001). β-casein (CASB_BOVIN) scored best among the caseins and had a relatively high score on total ACE inhibitory activity, while its score on peptide size was substantially lower. The same phenomenon was observed for α_{s1}-casein. Both proteins had relatively large, high ACE inhibitory peptides that were composed of smaller, less active peptides.

Table 3. Scoring of different proteins in pea, whey and casein by the ACE inhibitory peptide database.

Protein	Number of AA	Max. number of ACE inhibitory peptides	Score on highest ACE inhibitory activity		Score on shortest peptide sequence and then on highest activity	
			Score	Score/AA	Score	Score/AA
PEA						
vicilin	431	59	4.270	9.906	4.264	**9.892**
legumin J	481	72	3.394	7.056	3.009	6.255
legumin A2	498	76	2.781	5.584	2.771	5.564
legumin A	496	78	2.759	5.563	2.749	5.543
legumin B	544	78	3.502	6.438	2.879	5.292
legumin K	500	77	2.710	5.421	2.321	4.641
albumin PA2	231	43	2.401	**10.395**	1.014	4.391
vicilin 47 kDa	438	56	2.444	5.580	1.816	4.146
vicilin 14 kDa	124	14	0.403	3.248	0.403	3.248
convicilin	584	86	2.021	3.460	1.684	2.883
convicilin A	543	70	1.339	2.465	1.291	2.377
albumin PA1B	52	5	0.094	1.801	0.094	1.801
albumin PA1A	37	6	0.013	0.354	0.013	0.354
WHEY						
β-lactoglobulin	162	25	4.238	**26.160**	4.241	**26.181**
β_2-microglobulin	98	12	1.329	13.557	1.329	13.557
serotransferrin	685	105	10.825	15.803	6.995	10.211
lactotransferrin	689	121	17.950	26.052	5.463	7.929
BSA	583	60	4.489	7.699	4.176	7.163
proteose peptone	107	14	1.184	11.064	0.749	6.998
C5/C8						
lactoperoxidase	612	94	5.472	8.941	4.056	6.627
α-lactalbumin	123	14	0.814	6.614	0.814	6.614
lysozyme	130	20	0.546	4.203	0.492	3.781
$IgG_1/_2$ Hc precursor	470	63	3.107	6.611	1.620	3.446
proteose peptone C3	135	13	0.342	2.534	0.247	1.830
CMP	64	4	0.209	3.260	0.016	0.255

Table 3. Scoring of different proteins in pea, whey and casein by the ACE inhibitory peptide database (continued).

Protein	Number of AA	Max. number of ACE inhibitory peptides	Score on highest ACE inhibitory activity		Score on shortest peptide sequence and then on highest activity	
			Score	Score/AA	Score	Score/AA
CASEIN						
β-casein	209	32	3.402	**16.277**	0.947	**4.532**
α_{s2}-casein	207	28	0.709	3.425	0.708	3.422
α_{s1}-casein	199	37	2.159	10.851	0.562	2.822
κ-casein	169	19	0.636	3.763	0.147	0.869

The protein sequences with the highest scores for pea and whey were cleaved *in silico* by the gastrointestinal proteases pepsin, trypsin and α-chymotrypsin by means of the software program PeptideCutter (http://www.expasy.org/). Figure 3, 4 and 5 show the ACE inhibitory peptide sequences and the cleavage sites of the gastrointestinal enzymes in the amino acid sequences of albumin PA2, vicilin and β-lactoglobulin respectively.

For pea albumin PA2, 67 ACE inhibitory peptide sequences were found; as some of these were overlapping only 43 were detected in the scoring (Table 3, Figure 3). Most of these were dipeptides and had very potent ACE inhibitory activities: 77% of the peptides scored on highest ACE inhibitory activity demonstrated an IC_{50} value lower than 1000 µM and 40% had an IC_{50} value lower than 100 µM. The difference between the two scoring methods was due to the substitution of the tripeptide Leu-Gly-Pro (IC_{50} = 0.72 µM) by the more than 600 times less active dipeptide Gly-Pro (IC_{50} = 450 µM) when scoring on peptide size. Because of the large difference in ACE inhibitory activity, the substitution of only one peptide resulted in a substantial difference in score. By *in silico* hydrolysis, 69 peptide bonds could be cleaved by pepsin, 26 by trypsin and 50 by α-chymotrypsin, but some of the pepsin cleavage sites overlapped with the ones of α-chymotrypsin and trypsin. In total, 38% of the peptide bonds were cleaved by the gastrointestinal enzymes (at least 60% probability for trypsin and α-chymotrypsin). The less probable cleavage sites were interpreted as being cleaved in less than 60% of the protein molecules and left intact in the others. Four known ACE inhibitory dipeptides could be directly released by *in silico* digestion of albumin PA2: Leu-Tyr (IC_{50} = 18 µM), Gly-Ser (IC_{50} = 3800 µM), Gly-Lys (IC_{50} = 2500 µM) and Tyr-Trp (IC_{50} = 10 µM). Moreover, 19 precursors of ACE inhibitory peptide sequences were formed. By this way, at most 22 other ACE inhibitory peptides could be freed, the most active ones being Asp-Gly

(IC_{50} = 12 µM) (2 moles per mole of protein), Leu-Gly-Pro (IC_{50} = 0.72 µM), Met-Phe (IC_{50} = 45 µM) and Val-Tyr (IC_{50} = 10 µM).

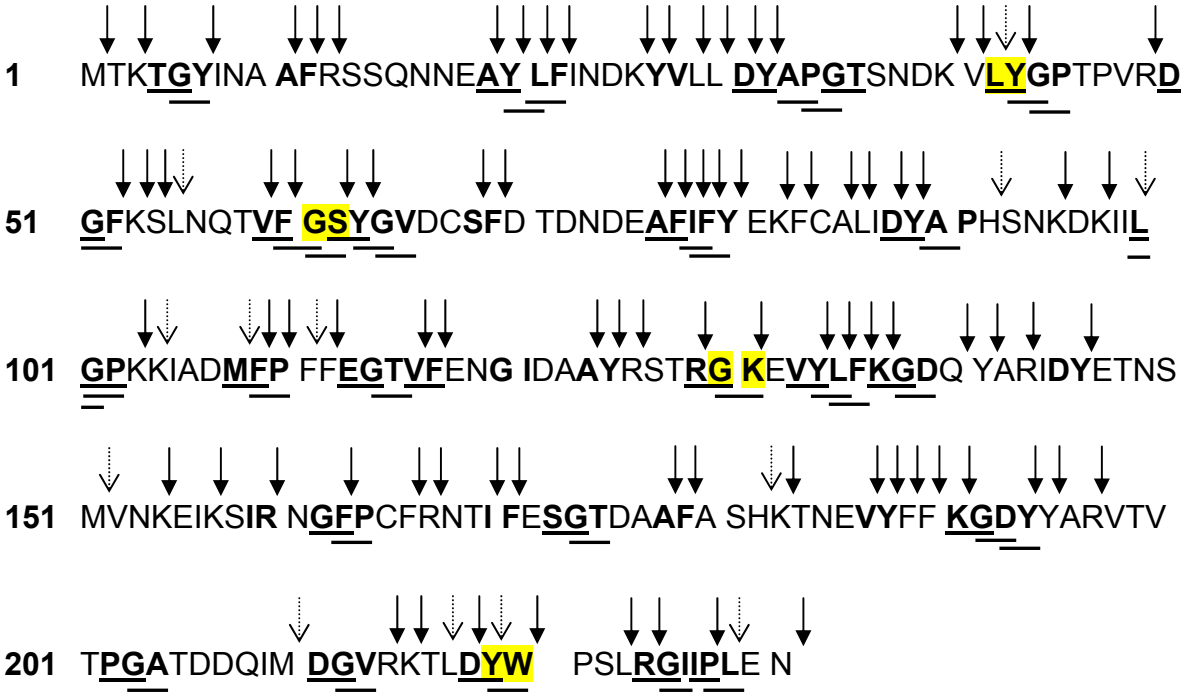

Figure 3. Amino acid sequence of albumin PA2 with the ACE inhibitory peptide sequences (**bold**, when overlapping: sequences underlined) and the cleavage sites of pepsin, trypsin and/or α-chymotrypsin (less than 60% probability cleavage sites for trypsin and α-chymotrypsin are indicated with dotted arrows). The ACE inhibitory peptides that are directly released are in yellow.

For pea vicilin, 88 ACE inhibitory peptide sequences were indicated compared to 59 in the scoring (Figure 4). All of the detected ACE inhibitory sequences were di- or tripeptides. Five tripeptides were composed of or overlapped with ACE inhibitory dipeptides, but only in the case of Phe-Gly-Lys (IC_{50} = 160 µM), the substituted dipeptide Phe-Gly (IC_{50} = 3700 µM) showed a lower ACE inhibitory activity. This explains the similar scores on highest ACE inhibitory activity and shortest peptide sequence. When scoring on highest ACE inhibitory activity, 64% of the ACE inhibitory peptide sequences had an IC_{50} lower than 1000 µM and 27% even possessed an IC_{50} lower than 100 µM. The most potent ACE inhibitory peptide sequence in vicilin was Leu-Lys-Pro with an IC_{50} of 0.32 µM. Pepsin could cleave vicilin at 112, trypsin at 52 and α-chymotrypsin at 74 positions. Vicilin had a theoretical digestibility of 37% (only cleavage sites with at least 60% probability for trypsin and α-chymotrypsin). Digestion of vicilin by gastrointestinal proteases predicted the release of 5 ACE inhibitory

dipeptides: Ile-Arg (IC_{50} = 696 µM), Gln-Lys (IC_{50} = 885 µM), Ser-Gly (IC_{50} = 8500 µM), Gly-Lys (IC_{50} = 2500 µM) and Ala-Phe (IC_{50} = 15.2 µM). Further degradation by brush border, intracellular and serum peptidases could free maximally 33 ACE inhibitory peptides from 25 precursor peptides. The most active peptides present in precursors were Ile-Tyr (IC_{50} = 2.1 µM) and Val-Lys (IC_{50} = 13 µM) (3 moles per mole of protein).

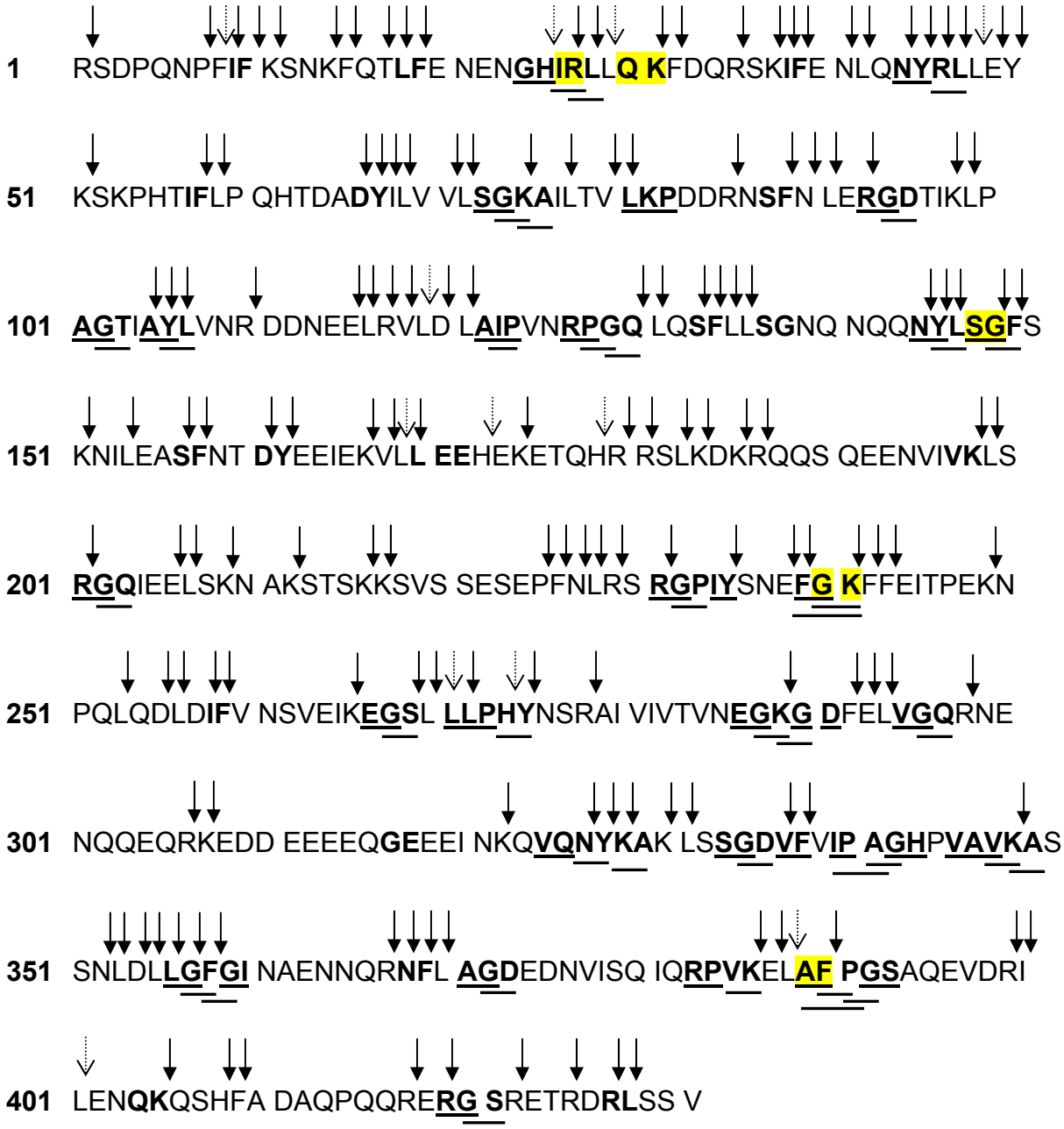

Figure 4. Amino acid sequence of vicilin with the ACE inhibitory peptide sequences (**bold**, when overlapping: sequences underlined) and the cleavage sites of pepsin, trypsin and/or α-chymotrypsin (less than 60% probability cleavage sites for trypsin and α-chymotrypsin are indicated with dotted arrows). The ACE inhibitory peptides that are directly released are in yellow.

Although the scoring was done with 25 non-overlapping ACE inhibitory peptide sequences, in total 50 different ACE inhibitory peptide sequences were present in the amino acid sequence of β-lactoglobulin (Figure 5). Some longer ACE inhibitory peptide sequences were present and four of them had a slightly higher ACE inhibitory activity than their partial peptides: Gly-Leu-Asp-Ile-Gln-Lys (IC_{50} = 580 µM), Val-Ala-Gly-Thr-Trp-Tyr (IC_{50} = 1682), Leu-Leu-Phe (IC_{50} = 80 µM) and Ala-Leu-Pro-Met-His-Ile-Arg (IC_{50} = 43 µM). This resulted in a slightly lower score on shortest peptide sequence compared to the one on highest ACE inhibitory activity. The very high score of β-lactoglobulin can be explained by the high number of ACE inhibitory peptides per amino acid and by the fact that 80% of the ACE inhibitory peptide sequences had an IC_{50} value lower than 1000 µM, while 36% of them exhibited an IC_{50} lower than 100 µM. The two most potent ACE inhibitory peptides present in the amino acid sequence of β-lactoglobulin were Lys-Trp (IC_{50} = 1.63 µM) and Leu-Lys-Pro (IC_{50} = 0.32 µM). In this whey protein, 37 cleavage sites for α-chymotrypsin, 48 for pepsin and 17 for trypsin were observed. In total, 39% of the peptide bonds were cleaved by the gastrointestinal enzymes (at least 60% probability for trypsin and α-chymotrypsin). Six ACE inhibitory peptides were directly produced by the combined action of pepsin, trypsin and α-chymotrypsin: Gln-Lys (IC_{50} = 885 µM), Ala-Leu-Pro-Met-His-Ile-Arg (IC_{50} = 43 µM), Ala-Leu-Pro-Met-His (IC_{50} = 521 µM), His-Ile-Arg (IC_{50} = 953 µM), Ile-Arg (IC_{50} = 696 µM) and Ser-Phe (IC_{50} = 130 µM). In addition, 8 precursor peptide sequences were released that contained 13 ACE inhibitory peptides, the most potent being Ile-Ala-Glu (IC_{50} = 35 µM).

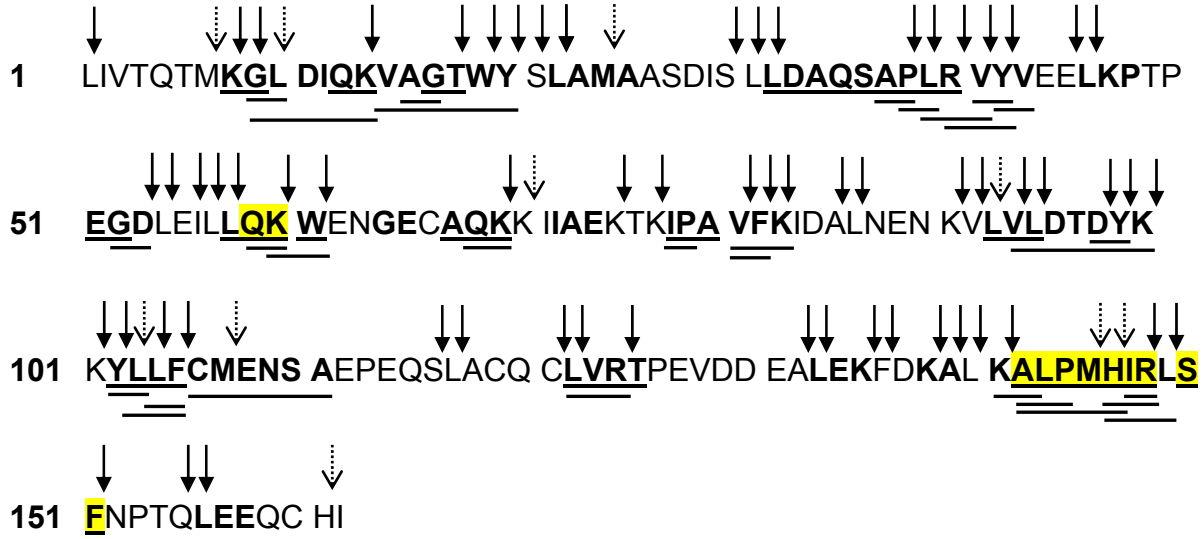

Figure 5. Amino acid sequence of β-lactoglobulin with the ACE inhibitory peptide sequences (**bold**, when overlapping: sequences underlined) and the cleavage sites of pepsin, trypsin and/or α-chymotrypsin (less than 60% probability cleavage sites for trypsin and α-chymotrypsin are indicated with dotted arrows). The ACE inhibitory peptides that are directly released are in yellow.

DISCUSSION

Gastrointestinal digestion constitutes a major barrier to the bioavailability of ACE inhibitory peptides (Pihlanto-Leppälä, 2001). In a previous study, high ACE inhibitory activity is obtained upon *in vitro* gastrointestinal digestion of pea and whey protein (Vermeirssen *et al.*, 2003b). In order to further elucidate the relative importance of the different enzymes and proteins in the release of ACE inhibitory peptides, the evolution of the ACE inhibitory activity during the *in vitro* physiological digestion of pea and whey protein was investigated. In addition, the stability of the ACE inhibitory digests to brush border peptidases was examined. Although in some studies the stability of isolated ACE inhibitory peptides towards gastrointestinal proteases is considered (Choi *et al.*, 2001; Kim *et al.*, 1999), the possible degradation by brush border peptidases is largely neglected. Protein degradation in the different phases of digestion was followed by SDS-PAGE and HPLC. This information was supplemented with theoretical data obtained by screening different protein sequences from pea and whey using an ACE inhibitory peptide database and data-mining software. Besides, these proteins were cleaved *in silico* by pepsin, trypsin and α-chymotrypsin. Hence, the theoretical release of ACE inhibitory peptides during gastrointestinal digestion could be indicated. The application of high throughput techniques in biology has increased the amount of information tremendously and the usefulness of databases has been demonstrated in this field (Vidal, 2001). Furthermore, this may change the way biological questions are formulated and addressed. In parallel, a database of ACE inhibitory peptide sequences may have important value in nutrition science. Instead of trying to isolate ACE inhibitory peptides blindly from food proteins by applying certain enzymes, a food protein known to contain several potent ACE inhibitory peptide sequences may be chosen to be digested by an enzyme that specifically releases these bioactive fragments.

Formation of ACE inhibitory activity over time

When the physiological digestion was studied over time and followed by a rat intestinal acetone extract digestion, the degree of proteolysis evolved differently than the ACE inhibitory activity. In the early stages of stomach, small intestine and mucosal phase digestion, the degree of proteolysis augmented first to level off in the later stage of the digestion phase. As the formed peptides and amino acids resided in the same medium as the protein and proteases, product inhibition may have occurred. Digestion in a dialysis bag or ultrafiltration membrane reactor may overcome this problem (Bordenave *et al.*, 2000; Gauthier *et al.*, 1986). The higher susceptibility to hydrolysis of pea protein, when compared to whey, was observed in the stomach and small intestine phase, but in the mucosal phase

the degree of proteolysis of the pea protein increased only slightly, while for the whey protein this increase was substantial. However, the increase in degree of proteolysis indicates only the release of 6% (w/v) TCA soluble peptides and amino acids from non-soluble proteins. As the brush border peptidases in the rat intestinal acetone extract tend to splice more oligopeptides rather than large proteins (Ganapathy and Leibach, 1999), the hydrolysis will not be reflected in the degree of proteolysis, but it will in the TNBS free α-amino-groups and this was the case in our experiments.

The ACE inhibitory activity of the pea protein reached 100% already after the first half hour of digestion, while for the whey protein this happened only in the small intestine phase. For the remainder of the digestion, 100% ACE inhibitory activity was maintained. Pihlanto-Leppälä *et al.* (2000) reported that pepsin hydrolysis for 3 h at pH 2 and 37°C in an E/S of 1/200 (w/w) is insufficient to release ACE inhibitory peptides from α-lactalbumin and β-lactoglobulin and that trypsin is necessary for the formation of high ACE inhibitory activity from whey proteins. Mullally *et al.* (1997a), on the other hand, found a similar and rather high percent ACE inhibitory activity after digestion of whey proteins with pepsin at pH 3 or trypsin at pH 8 or the combination for a total of 4 h at 50°C in an E/S of 3/1000 (w/w). When characterising goat whey peptic hydrolysate, Bordenave *et al.* (2000) concluded that a residence time of 1 h in an ultrafiltration reactor at pH 2 and 40°C is enough to ensure total degradation of α-lactalbumin by pepsin in an E/S of 1/50 (w/w). From the evolution of the ACE inhibitory activity of the whey protein in the stomach phase it is very likely that a longer incubation time with pepsin could lead to 100% ACE inhibitory activity. However, to obtain a low IC_{50}, additional hydrolysis by trypsin and α-chymotrypsin would be essential, as is indicated by the large drop in IC_{50} from the end of the stomach to the small intestine phase. Remarkably, the decrease in IC_{50} during this digestion phase was more pronounced for whey, while at the same time this protein displayed a smaller increase in the degree of proteolysis. The latter supports the observation of Mullally *et al.* (1997a) that there is no direct relationship between the degree of proteolysis and the ACE inhibitory activity. At the end of the small intestine phase, the IC_{50} of the pea digest was also significantly lower than at the end of the stomach phase, but the change was not as extreme as for the whey protein. As the pea and whey physiological digest were hydrolysed by brush border peptidases, the IC_{50} value increased again, signifying that degradation of ACE inhibitory peptides started to dominate or that conversion into less active ACE inhibitory peptides occurred, diluting the total activity. Moreover, the ACE present in the rat intestinal extract could have resulted in an underestimation of the ACE inhibitory activity. Considering a 25% overestimation of the IC_{50} value, this may explain the higher IC_{50} after the brush border phase compared to the small intestine phase for the pea protein. However, for the whey protein, the more substantial

increase of the IC_{50} after the brush border phase cannot be explained solely by the presence of ACE in the sample. Nevertheless, high ACE inhibitory activity was still present at the end of the digestion, both for pea and whey protein, increasing the potential for these hydrolysates to reduce the blood pressure *in vivo*.

Follow-up of the protein degradation over time

SDS-PAGE visualised the proteins with a molecular weight of 14 to 94 kDa present in the proteins. Because β-mercapto-ethanol disrupts disulphide bounds, not only the albumin fraction, but also the globulin fraction of the pea protein was visible on the gel. The high sensitivity of the pea protein for hydrolysis was noticeable as well: after the stomach phase, bands with molecular weight higher than 20 kDa faded away, and after the small intestine and mucosal phase only a light spot around 10 kDa remained, indicating that most proteins were degraded to small peptides. Convicilin, PA2 and to a lesser extent vicilin and legumin β seemed to resist the stomach digestion, but all these proteins were completely broken down during the subsequent phases of *in vitro* digestion. Convicilin, vicilin and legumin α are reported to be sensitive to hydrolysis, while legumin β due to its highly ordered structure and high hydrophobicity resists enzyme hydrolysis (Crevieu *et al.*, 1997; Nielsen *et al.*, 1988). Albumins are on average less digestible than globulins, with PA2 having a relatively high cysteine content and consequently a lot of disulphide bonds (Crevieu *et al.*, 1997).

For the whey protein, α-lactalbumin was degraded during the stomach digestion, while the band of β-lactoglobulin remained present untill the end of digestion. β-lactoglobulin resists pepsin and largely trypsin hydrolysis (Guo *et al.*, 1995), while it is hydrolysed by α-chymotrypsin to a limited extent (Schmidt and Poll, 1991). Bovine serum albumin and the immunoglobulin fraction were also broken down during the stomach phase. Although some bovine immunoglobulins appear in the ileum of human adults after oral ingestion of an immunoglobulin concentrate (Roos *et al.*, 1995), they seemed to be completely degraded in the *in vitro* physiological digestion of whey protein isolate. In a study where the hydrolysis of whey protein concentrate is followed by gel filtration, trypsin and to a lesser extent α-chymotrypsin degrade the four major whey proteins, immunoglobulins, BSA, β-lactoglobulin and α-lactalbumin in relatively large molecular weight fractions (Angelo *et al.*, 1982). This is confirmed by the appearance of the three bands, which are assumed to be fragments of β-lactoglobulin, after the small intestine phase.

In silico screening of the ACE inhibitory peptide potential and release

Protein sequences from pea and whey protein were obtained from the protein databases SwissProt, TrEMBL and NCBI. For pea, each type of protein is present in the database and for some even different genetic variants are reported. For whey, the complete amino acid sequences of the major proteins and some minor enzymes are also found. These protein sequences were screened by the ATO ACE inhibitory peptide database, containing 498 ACE inhibitory peptide sequences with their IC_{50} value from literature, by means of the data-mining software. A database of biologically active peptide sequences has already been reported by Dziuba *et al.* (1999b), although it consisted of only 201 ACE inhibitory peptides. This is however the first study to attribute ACE inhibitory peptide potential scores to proteins and to investigate the *in silico* release of these ACE inhibitory peptide sequences.

The different proteins were scored on ACE inhibitory activity in two ways. This provided information on the theoretical content of ACE inhibitory peptide sequences and whether they included some smaller active fragments. The latter is important as di- and tripeptides are more easily absorbed intact (Webb, 1990). In order to facilitate the interpretation of these scores, the caseins were also screened by the database, as a lot of potent ACE inhibitory peptides have already been released from these proteins (Fitzgerald and Meisel, 2000). This scoring was confirmed for the proteins albumin PA2, vicilin and β-lactoglobulin, respectively the pea and whey proteins with the highest scores. The scores of all pea globulins were in the narrow range of 2 to 10. This may be explained by the fact that the amino acid sequence of all pea globulins shows some similarity. Vicilins and legumins have evolved from the duplication of the same ancestral gene, but in legumins a novel N-terminal domain has been recruited somewhere in evolution (Gibbs *et al.*, 1989). Convicilin on the other hand, differs only from vicilin by the insertion of a 121 amino acid sequence near the N-terminus of the protein (Bown *et al.*, 1988). β-lactoglobulin, the major whey protein, and lactotransferrin had the highest scores on ACE inhibitory activity. The latter is known to exert different biological activities like antibacterial, immunomodulatory, anti-tumour and antioxidant activity and to contain a number of bioactive peptide sequences with antimicrobial activity (Baker *et al.*, 2002). β-casein and α_{s1}-casein scored higher on ACE inhibitory activity than the pea proteins and α-lactalbumin and BSA, but much lower than β-lactoglobulin. Caseins are known to contain a lot of proline, which may provide resistance to complete degradation by human proteases. Though, the proline content cannot be related to high ACE inhibitory potential of a protein: although bovine serum albumin contains as much proline as β-casein, it had a lower ACE inhibitory activity score. However, it must be taken into account that the scoring was performed by means of IC_{50} from literature, that were probably in some cases obtained by different ACE inhibition assays, which may result in slightly different values. Moreover, the

database can only search for the ACE inhibitory peptide sequences it contains. As this database was very recently updated and contained a substantial amount of ACE inhibitory peptides, its predictive value is significant. Hence, this database is a powerful tool to screen the ACE inhibitory potential of proteins, provided their amino acid sequence is available.

Subsequently, the proteins were hydrolysed *in silico* by pepsin, trypsin and α-chymotrypsin. This procedure possessed some limitations as well. During gastrointestinal digestion, proteins are first cleaved by pepsin in the stomach and then by trypsin and α-chymotrypsin in the small intestine. During the *in silico* digestion, these three proteases cleaved at the same time, which means that neighbouring amino acids that were eliminated by the action of pepsin, may have exerted some favourable or unfavourable effects on the cleavage by trypsin and α-chymotrypsin *in silico*. Furthermore, the program takes a specified cleavage preference into account. This may not include all the exceptions that occur upon the presence of a certain amino acid in a certain position relative to the spliced peptide bond. Finally and most importantly, the protein conformation is totally neglected during *in silico* digestion. This may result in a higher number of cleavages predicted compared to the *in vitro* or *in vivo* situation. *In silico*, the digestibility of albumin PA2, vicilin and β-lactoglobulin was very similar. During *in vitro* digestion, however, the pea proteins are more easily degraded by pepsin than β-lactoglobulin. This is due to the compact native structure of β-lactoglobulin (Kinsella and Whitehead, 1989). Hydrophobic amino acid residues forming bonds susceptible to proteolysis by pepsin, are buried inside the molecule and are inaccessible for the enzyme. Overall, the *in silico* digestion is more pronounced than in reality, because it indicates where the protein can be hydrolysed. The *in vitro* and *in vivo* digestion conditions determine whether the hydrolysis at a certain position in the protein sequence will occur or not. For example, β-lactorphin, Tyr-Leu-Leu-Phe, is released from β-lactoglobulin upon *in vitro* digestion by pepsin, trypsin and α-chymotrypsin (Antila *et al.*, 1991). During the *in silico* digestion of β-lactoglobulin, however, this peptide was degraded. The lactokinin Ala-Leu-Pro-Met-His-Ile-Arg was theoretically released from β-lactoglobulin. Some minor degradation by α-chymotrypsin took place, which is confirmed by experimental data (Mullally *et al.*, 1997b) and by our study on the *in vitro* gastrointestinal digestion of this peptide (Chapter 3). However, the major degradation product formed, His-Ile-Arg, is also ACE inhibitory active (Mullally *et al.*, 1997b). The RP-HPLC chromatograms of the physiological whey digest did not exclude the release of the heptapeptide or its α-chymotrypsin degradation products. In the study of Pihlanto-Leppälä *et al.* (1997), only digestion of β-lactoglobulin by trypsin releases the heptapeptide, while digestion by a combination of pepsin, trypsin and α-chymotrypsin releases His-Ile-Arg. The period of digestion here is 24 h, which is very long. The same authors detect the ACE inhibitory active fragment Ala-Leu-Pro-Met-His and the

fragment Leu-Pro-Met-His-Ile-Arg in the digest of β-lactoglobulin obtained by 3 h pepsin, then 7 h trypsin and α-chymotrypsin hydrolysis (Pihlanto-Leppälä *et al.*, 2000). Other ACE inhibitory peptides released in this study are Cys-Met-Glu-Asn-Ser-Ala and Val-Leu-Asp-Thr-Asp-Tyr-Lys. Moreover, the fragment Val-Ala-Gly-Thr-Trp is found. RP-HPLC combined with mass spectrometry of the pea and whey digests could confirm the formation of the theoretically released ACE inhibitory peptide sequences during the *in vitro* physiological digestion in our study.

When combining the theoretical data with our experimental results, the evolution in IC_{50} during the physiological digestion can be related to the ACE inhibitory potential of the degraded proteins. For pea, most proteins were already broken down to molecular masses lower than 10 kDa after the stomach phase and were further degraded during the small intestine phase. All these proteins had similar scores in the *in silico* screening. This corresponds to the high ACE inhibitory activity already observed after the stomach phase and which further increased in the small intestine phase. The protein with the highest score on ACE inhibitory activity and the major whey protein, β-lactoglobulin, was only degraded from the small intestine phase on, theoretically releasing ACE inhibitory peptides like Ala-Leu-Pro-Met-His-Ile-Arg. This is consistent with the fact that the IC_{50} value of whey protein decreased substantially during hydrolysis with trypsin and α-chymotrypsin. Hence, for whey, it could be useful to concentrate on the release of ACE inhibitory peptides from β-lactoglobulin.

In conclusion, this paper demonstrates the evolution of the ACE inhibitory activity during physiological digestion followed by a brush border phase. It further indicates how the IC_{50} and the protein degradation can be related to each other by means of an ACE inhibitory peptide database.

ACKNOWLEDGEMENTS

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CHAPTER 7

FRACTIONATION, *IN VITRO* INTESTINAL TRANSPORT AND ANTIHYPERTENSIVE ACTIVITY OF PEA AND WHEY DIGESTS

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Chapter 7

Fractionation, in vitro intestinal transport and antihypertensive activity of pea and whey digests

ABSTRACT

The ACE inhibitory activity of pea and whey hydrolysate, obtained by *in vitro* gastrointestinal digestion, was further increased upon purification by ultrafiltration-centrifugation and RP-HPLC. The most active RP-HPLC fractions of the permeates had IC_{50} values as low as 0.016 mg P (protein)/ml for pea and 0.003 mg P/ml for whey, which signifies that the ACE inhibitory activity of the pea digest was more than 4 times enriched, while that of the whey digest was more than 13 times augmented. The intestinal transport of active compounds in the digests and permeates was investigated in Caco-2 cell monolayers. Although the samples retained relatively high ACE inhibitory activity after 2 h incubation in the presence of Caco-2 homogenates, no or only little ACE inhibitory activity was detected in the basolateral compartments of the Caco-2 cell monolayers after 1 h transport experiment, even after concentration of the samples. Some ACE inhibitory activity was observed in the transport experiments at high concentrations (45 mg P/ml digest, 50 mg P/ml permeate), but these were associated with compromised cell monolayer integrity as indicated by decreased TEER and increased sodium fluorescein fluxes. Taking into account that the Caco-2 model is tighter than intestinal mammalian tissue and that the ACE inhibitory activity of pea and whey hydrolysates resisted intestinal peptidase activity well, absorption of these peptides in substantial quantities might still take place *in vivo*. After intravenous administration of a dose of 50 mg P/kg BW in spontaneously hypertensive rats (SHR), pea permeate exerted a transient, but strong antihypertensive effect of 44.4 mmHg. As it was immediately followed by an increase in blood pressure above the initial value, this result has to be interpreted with caution and further research is necessary. Whey permeate exerted no effect at a dose of 50 mg P/kg BW.

Keywords: ACE inhibitory peptides, Caco-2, intestinal transport, SHR

INTRODUCTION

Angiotensin I converting enzyme (ACE, EC 3.4.15.1) inhibitory peptides may result in a lowering of the blood pressure by inhibition of the ACE enzyme, which results in a decrease of the vasoconstrictor angiotensin II and an increase in the vasodilator bradykinin (Eriksson *et al.*, 2002). ACE inhibitory peptides have already been found in several food protein hydrolysates and ferments (Dziuba *et al.*, 1999; Yamamoto, 1997). Foods containing ACE inhibitory peptides have shown to be effective in both the prevention and treatment of hypertension (Sipola *et al.*, 2002; Takano, 1998). As ACE inhibitory peptides have not revealed any side effects yet, they may represent an alternative for or an additional treatment to antihypertensive drugs and may be applied in the prevention of a high blood pressure as well (Fitzgerald and Meisel, 2000).

Purification of ACE inhibitory active food protein hydrolysates may lead to more active fractions or ultimately a potent ACE inhibitory peptide. The studies of Mullally *et al.* (1997) and Pihlanto-Leppälä *et al.* (2000) suggest that ultrafiltration may be exploited to enrich ACE inhibitory peptides from whey proteins. Additional purification by ion chromatography and RP-HPLC augments substantially the ACE inhibitory activity from wheat germ hydrolysate: the IC_{50} value of the digest is 0.67 mg/ml, while the IC_{50} of the pure peptide Ile-Val-Tyr is 0.48 µM or 0.189 µg/ml (Matsui *et al.*, 1999).

ACE inhibitory peptides exert an antihypertensive effect *in vivo* if they reach the cardiovascular system in an active form. Upon oral administration, they therefore need to resist complete degradation by gastrointestinal proteases and brush border peptidases and they have to be absorbed through the intestinal wall with preservation of their physiological activity. During this oral delivery route, degradation of peptides can take place, which results in an activation or inactivation of their biological activity (Fitzgerald and Meisel, 2000; Pihlanto-Leppälä, 2001). This partially explains the discrepancy between the ACE inhibitory activity and the antihypertensive effect after oral administration of a food protein hydrolysate, as observed in the study of Abubakar *et al.* (1998). Few studies on ACE inhibitory peptides consider the stability during digestion and absorption in the gastrointestinal tract. After oral administration of Calpis™ sour milk, containing the antihypertensive peptides Val-Pro-Pro and Ile-Pro-Pro, these peptides are detected in the abdominal aorta of spontaneously hypertensive rats (SHR), suggesting that these casokinins are directly absorbed without being decomposed by the digestive enzymes (Masuda *et al.*, 1996). When the transport of the antihypertensive peptide Val-Pro-Pro is investigated in Caco-2 cell monolayers, which are a representative model of the human intestinal epithelial cell barrier (Wilson *et al.*, 1990), uptake of the intact peptide is suggested to occur by the paracellular route (Satake *et al.*,

2002). After a single oral administration of the ACE inhibitory dipeptide Val-Tyr, this peptide is dose-dependently detected in the plasma of normotensive and mild hypertensive subjects (Matsui *et al.*, 2002a; Matsui *et al.*, 2002b). Although it exerts a 10% systolic blood pressure reduction after intravenous administration of 50 mg/kg BW in SHR (Matsufuji *et al.*, 1995), it fails to exert an antihypertensive effect at a single oral dose of 12 mg in mild hypertensive subjects (Matsui *et al.*, 2002b).

In previous studies, high ACE inhibitory activity is produced by *in vitro* gastrointestinal digestion of pea and whey protein (Vermeirssen *et al.*, 2003a; Vermeirssen *et al.*, 2003b). An *in vitro* digestion simulating the physiological conditions *in vivo* is adequate for the formation of maximal ACE inhibitory activity. Moreover, whey hydrolysates are more ACE inhibitory active than pea hydrolysates (Vermeirssen *et al.*, 2003b). The aim of this study was to obtain more active fractions from pea and whey protein hydrolysates by means of ultrafiltration and RP-HPLC. Subsequently, the intestinal absorption of the active compounds present in the digests and permeates obtained by ultrafiltration was investigated in the Caco-2 cell system. Finally, the permeates were injected intravenously in SHR to measure potential antihypertensive effects.

MATERIALS AND METHODS

Products

The products used during *in vitro* gastrointestinal digestion and HPLC analysis are described in Chapter 5. Transport buffer (pH 7.4) consisted of Hanks' Balanced Salt Solution (HBSS) supplemented with HEPES (both from Invitrogen Life Technologies, Carlsbad, CA, USA) and D-glucose (G 7021, Sigma-Aldrich, St.-Louis, MO, USA) at final concentrations of 10 mM and 25 mM, respectively. When explicitly mentioned, transport buffer at pH 6 was used, which contained HBSS supplemented with 10 mM MES (Invitrogen Life Technologies) and 25 mM D-glucose. Dulbecco's Modified Eagle Medium (DMEM) containing GlutaMAX™, non-essential amino acids, penicillin (10 000 IU/ml), streptomycin (10 000 µg/ml) and fetal bovine serum were also from Invitrogen Life Technologies. Non-specified products were analytical grade from VWR International (Zaventem, Belgium).

In vitro gastrointestinal digestion

The experimental conditions of the *in vitro* physiological digestion are mentioned in Chapter 5.

ACE inhibitory activity

The treatment of the samples is described in Chapter 4. ACE inhibition assay 3 in Chapter 2 was used to determine the ACE inhibitory activity.

Protein content

Protein content was determined by the Bio-Rad DC protein standard assay (Bio-Rad Laboratories S.A.-N.V., Nazareth Eke, Belgium) with BSA as standard, based on the method of Lowry (Lowry *et al.*, 1951), according to the procedures of the manufacturer.

Ultrafiltration-centrifugation

Eighty milligram lyophilised digest was dissolved in 2 ml milliQ water (Millipore, Bedford, MA, USA) and ultrafiltrated-centrifuged in Centricon YM-3000 tubes (MWCO = 3000 Da) (Millipore) (low adsorption, hydrophilic, regenerated cellulose membranes) for 2 h at 7500 g. Permeate and retentate were lyophilised and dissolved in demineralised water or buffer to determine the ACE inhibitory activity.

HPLC

The permeate was fractionated using preparative RP-HPLC by injecting repetitively 1 ml of a 35 to 40 mg P (protein)/ml solution on a Vydac C_{18} column (250 x 10 mm) and ABI Model 783A Gradient Controller and Absorbance Detector at 210 nm with an ABI Model 400A Solvent Delivery System and 491 Dynamic Mixer/Injector (Applied Biosystems, Foster City, CA, USA) operating at room temperature. The flow rate amounted to 4 ml/min. A linear gradient from 95% solvent A (H_2O + 0.1% (w/v) TFA) to 40% solvent B (acetonitrile + 0.085% (w/v) TFA) in 30 min was applied, thereafter the gradient increased linearly to 95% solvent A in 10 min and remained at 95% solvent A during the last 10 min. Two minutes after the start of the elution, fractions were collected every 6 min (Figure 1). The five fractions were lyophilised and dissolved in demineralised water to determine the ACE inhibitory activity.

The profiles of the permeates were obtained by analytical RP-HPLC by injecting 100 µl of a 10 mg P/ml solution on a Prosphere 300 Å C_{18} column (250 x 4.6 mm, 5 µm) (Alltech Associates, Deerfield, IL, USA) and a Dionex (Sunnyvale, CA, USA) HPLC with an autosampler ASI-100, pump series P580, STH585 column oven, UV-VIS detector UVD340S operating at 210 nm and Chromeleon 6.0 software. The same programs as for the preparative RP-HPLC were applied.

Caco-2 cell culture

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in 75 cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) in an atmosphere of 5% CO_2 and 90% relative humidity at 37°C. Cells were passaged every 3-4 days (at 70-80% confluence) at a split ratio of 1:7. The Caco-2 cells were maintained in high glucose (4.5 g/l) DMEM containing glutaMAX™, 100 U/ml of penicillin, 100 µg/l of streptomycin, 1% (v/v) non-essential amino acids and 10% (v/v) fetal bovine serum. Cells were negative for *Mycoplasma* infection.

Stability experiments with Caco-2 homogenates

The Caco-2 homogenates were prepared as described in Chapter 3. Pea and whey digest and permeate at a concentration of 1 mg P/ml dissolved in transport buffer were incubated with the Caco-2 homogenates in an E/S ratio of 1/6 (w/w) at 37°C. The IC_{50} value was determined before the experiment, at the start, after 1 and 2 h. The percent ACE inhibitory activity was also assessed after 0.5 h and 1.5 h. A control sample containing only the Caco-2 homogenate in transport buffer was also analysed for ACE activity and ACE inhibitory activity.

Caco-2 transport study

For the transport studies, Caco-2 cells were plated at a density of 40 000 cells/cm^2 on Transwell membrane inserts (3 µm pore diameter, 12 mm diameter, Costar, Cambridge, MA, USA). Confluence was reached within 3-4 days after seeding and the monolayers (cell passage 90-110) were used for the experiments between days 18-24 post-seeding. The Caco-2 cell system was validated as described by Augustijns *et al.* (1998). Before the experiments, the monolayers were rinsed with transport buffer and preincubated for 30 min at 37°C. Thereafter, transepithelial electrical resistance (TEER) of the monolayers was measured at 37°C using a Millicel ERS apparatus (Millipore) to check cell monolayer integrity. Only monolayers with TEER values above 300 $\Omega.cm^2$ were used in the experiments. The transport buffer was then replaced by 0.5 ml transport buffer containing the test compound (pH 7.4 or pH 6) at the apical or mucosal side, while 1 ml transport buffer (pH 7.4) was added to the basolateral or serosal side. After 1 h incubation at 37°C, the basolateral medium was withdrawn and analysed for ACE inhibitory activity. The TEER was measured again and 1.5 ml fresh transport medium was added to the basolateral side, while the apical compartment was replaced by a solution of sodium fluorescein (0.5 ml; 1 mg/ml). After 1 h incubation at 37°C, the basolateral medium was collected and analysed for sodium

fluorescein transport. Sodium fluorescein was used as a hydrophilic marker for cell monolayer integrity. Typical sodium fluorescein flux values, measured by UV absorption of the basolateral solution at 490 nm, across Caco-2 monolayers after the transport experiment should be lower than 1% h^{-1} (Augustijns *et al.*, 1998). Donor and basolateral samples were both analysed for ACE inhibitory activity, immediately and in the basolateral samples also after concentrating (usually 4 times) by lyophilisation. The effect of concentrated transport buffer on the ACE inhibitory activity of the concentrated samples was taken into account. The following solutions were supplemented to the apical side of the Caco-2 cell monolayer: pea and whey digest at 45 and 9 mg P/ml, pea and whey permeate at 10 mg P/ml, pea digest heat treated at 98°C for 5 min at 33 mg P/ml. In one experiment, transport buffer at pH 6 was applied to the apical compartment and the transport of the following samples was investigated: pea digest heat treated at 98°C for 5 min at 45 mg P/ml, pea and whey digest at 9 mg P/ml, pea permeate at 10 mg P/ml, pea and whey permeate at 50 mg P/ml. Results are presented as mean ± standard error (SE) (if more than one experiment) of two to four experiments, with two to three repetitions for each condition per experiment.

Osmolarity

The osmotic concentration was determined by measuring the freezing point depression on a model 3D3 Osmometer (Advanced Instruments, Inc., Norwood, MA, USA).

SHR experiments

SHR, 12 weeks old, were supplied by Janvier (Le Genest-St.-Isle, France). Twenty-one SHR (290-345 g) with tail SBP of > 180 mmHg were used, and were divided in 4 groups: 6 control animals and 5 animals in the other groups. The SHR was anesthetised with 1 ml/kg Nembutal (60 mg/ml pentobarbital) (Sanofi Santé Animale, Brussel, Belgium), and the femoral artery was cannulated for recording the mean arterial blood pressure with a Druck Blood Pressure Transducer PDCR 75/1 (Orecon, Leuven, Belgium) and Kipp & Zonen BD40 pen recorder (Kipp & Zonen B.V., Delft, The Netherlands). PE50 and PE10 type catheters were used for the femoral artery and vein, respectively. The rats were allowed to stabilise for 5 min after the surgery. Pea and whey permeate, dissolved in physiological saline, were injected at a dose of 50 mg P/kg BW in the femoral vein. Physiological saline (500 µl) and captopril dissolved in physiological saline (5 mg/kg BW) were used as negative and positive controls, respectively. All experiments were carried out in accordance to national guidelines.

Statistical analysis

All values are reported as mean ± standard error of the mean (n_{min} = 3). A Student t-test was used to compare the ACE inhibitory activity (log IC_{50}) of the permeate and the retentate with the one of the digest. It was also investigated if the collected fractions exhibited a higher or lower ACE inhibitory activity than the permeate. For the SHR experiments, the blood pressure after injection was compared to the initial blood pressure by a Student t-test.

RESULTS

Fractionation of pea and whey digests

A 44.4 mg/ml pea and whey protein solution (90% protein content) exhibited an IC_{50} value of 16 mg/ml and 18 mg/ml, respectively. An *in vitro* physiological digestion that simulated the conditions of protein digestion *in vivo*, produced hydrolysates from pea and whey protein with high ACE inhibitory activity. The IC_{50} values amounted to 0.076 mg/ml and 0.048 mg/ml, respectively (see Chapter 5). The protein content of pea and whey digest was 92 ± 2% and 86 ± 3%, respectively.

A first enrichment of ACE inhibitory peptides was obtained by ultrafiltration-centrifugation with a MWCO of 3000 Da. The permeate, the low molecular weight fraction, was highly hygroscopic and its ACE inhibitory activity was more accurately expressed relative to the protein content. A further purification of this permeate was performed by RP-HPLC. Figure 1 shows the RP-HPLC profile of the pea and whey permeate and indicates the 5 different fractions that were collected. The first fraction remained a gel even after extensive lyophilisation. For this reason and, because only a small amount of sample was wasted for protein determination, the ACE inhibitory activity was expressed relative to the protein content for comparison of the different samples. For the whey permeate sharp individual peaks could be observed, while for the pea permeate on the other hand, most of the peaks merged.

An overview of the different ACE inhibitory activities obtained by ultrafiltration-centrifugation of the digests and RP-HPLC fractionation of the permeates is depicted in Figure 2. The permeate of both pea and whey had a higher ACE inhibitory activity than the digest, while the IC_{50} value of the retentate did not differ significantly from the corresponding digests' IC_{50}. The former effect was more pronounced for whey, where the IC_{50} value in the molecular weight fraction lower than 3000 Da was one third of the IC_{50} of the digest. A further increase in ACE inhibitory activity was obtained by RP-HPLC fractionation of the permeate. Both for pea and whey protein, the ACE inhibitory activity in the first fraction was lower than in the permeate. In fraction II, III an IV of the pea permeate a higher ACE inhibitory activity was observed. The lowest IC_{50} value of 0.016 mg P/ml was found in fraction IV, which was more than four times lower than the IC_{50} value of pea digest. For whey, the fractionation resulted in an even higher enrichment of ACE inhibitory activity. Fraction III, IV and V had higher ACE inhibitory activities compared to the permeate. The lowest IC_{50} value, 0.003 mg P/ml found in fraction III, was more than thirteen times lower than the IC_{50} value of the whey digest.

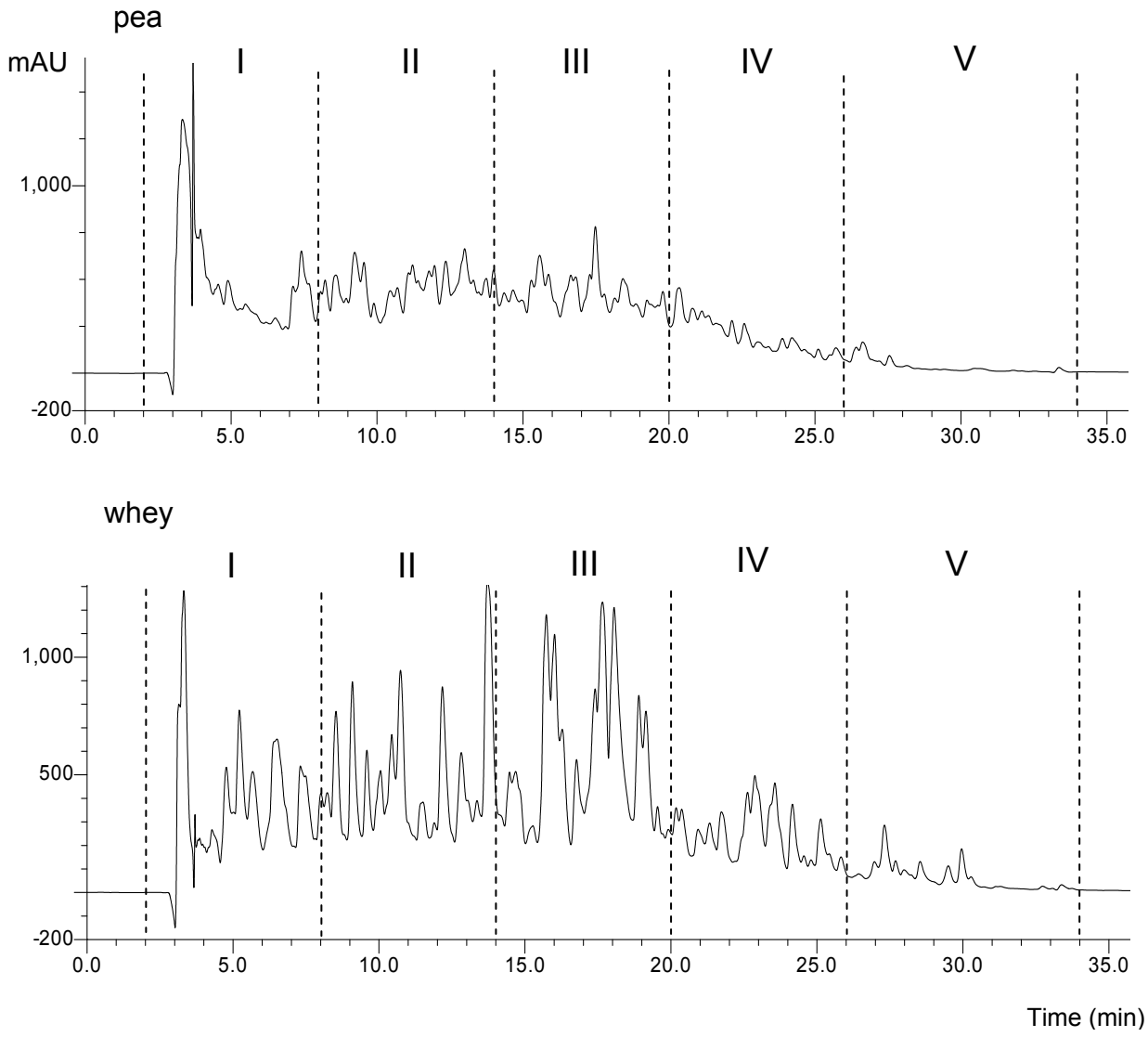
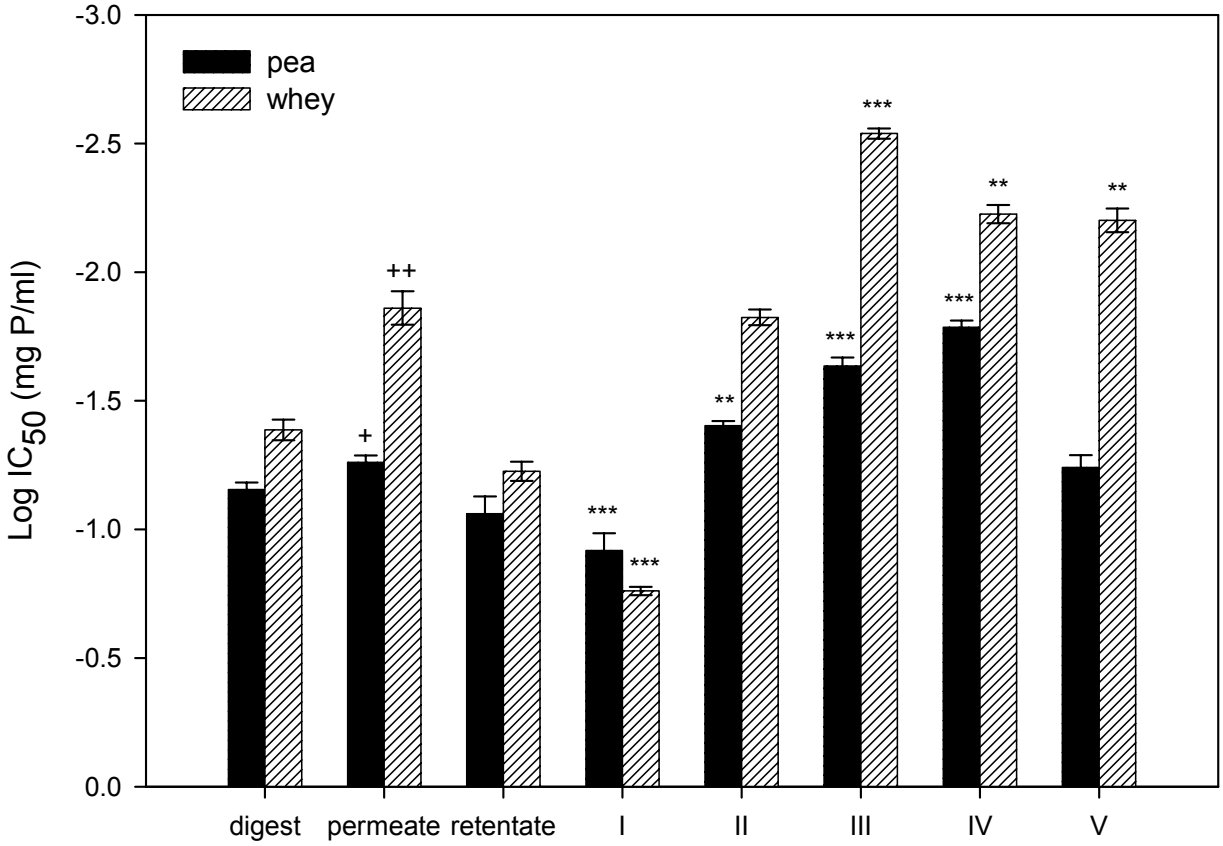


Figure 1. HPLC profile of pea and whey permeate and the different fractions (I, II, III, IV, V) that were collected.

The yield of the three most active fractions was estimated for pea and whey protein (Table 1). For the fractions II, III and IV from pea, a yield of about 5% was observed. For the fractions III, IV and V from whey, the yield was approximated 2%.

Table 1. Estimated yields (g) of digest, permeate and the three most active fractions from 4% (w/v) pea and whey.

	Pea		Whey	
Protein	4.44 **g**	4 **g P**	4.44 **g**	4 **g P**
Digest	3.50	3.20	4.44	3.81
Permeate	1.18	0.61	1.23	0.51
Fractions	0.25	0.19	0.11	0.08



IC_{50} mg P/ml								
Pea :	0.070	0.055	0.087	0.121	0.040	0.023	0.016	0.057
Whey :	0.041	0.014	0.059	0.173	0.015	0.003	0.006	0.006

Figure 2. Log IC_{50} and IC_{50} (mg P/ml) of the pea and whey digest, permeate and retentate and the fractions I, II, III, IV and V collected after RP-HPLC fractionation of the permeate (n_{min} = 3).
Significantly different from the digest: +p < 0.05, ++p < 0.01.
Significantly different from the permeate: *p < 0.05, **p < 0.01, ***p < 0.001.

Stability experiments with Caco-2 homogenates

A 1 mg P/ml solution of pea and whey digest and permeate respectively, was incubated at 37°C in the presence of Caco-2 homogenates. At regular intervals, the ACE inhibitory activity of these solutions was analysed and the IC_{50} was determined before the experiment, immediately after the addition of the Caco-2 homogenates and after 1 and 2 h incubation (Figure 3). The ACE inhibitory activity decreased, and consequently the IC_{50} value increased, in all solutions over time. The ACE inhibitory activity diminished substantially more in whey than pea digests and permeates. At the end of the experiment, the 1 mg P/ml pea digest still

retained 90% of its initial ACE inhibitory activity, while for the pea permeate this was 89%. The activities of the whey digest and permeate reduced to 72% and 69%, respectively.

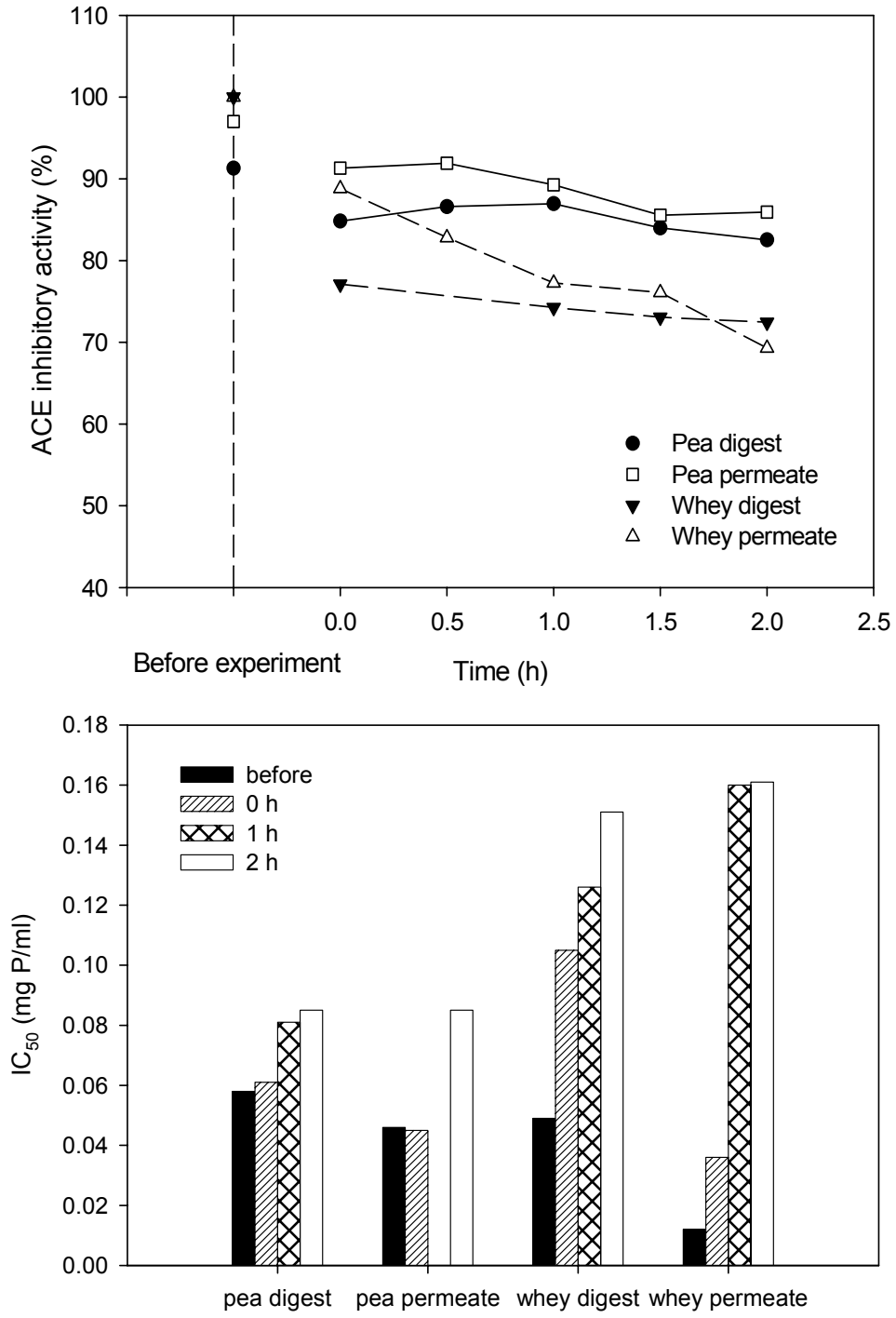


Figure 3. ACE inhibitory activity (%) of a 1 mg P/ml solution and IC_{50} value (mg P/ml) of the digest and the permeate of pea and whey protein before and during incubation with Caco-2 homogenates.

For the pea digest the IC_{50} value increased one and a half times, while the IC_{50} value of the pea permeate almost doubled upon 2 h incubation with Caco-2 homogenates. The IC_{50}

value of the whey digest at the end of the experiment was three times its initial value. The most pronounced effect was observed for the whey permeate, for which the IC_{50} value augmented thirteen times. Nevertheless, all samples still had high ACE inhibitory activity after 2 h incubation with Caco-2 homogenates. Solutions with only Caco-2 homogenates failed to show any ACE activity or significant ACE inhibitory activity.

Caco-2 transport study

In a first set of experiments, the intestinal transport of pea and whey digest at 45 and 9 mg P/ml concentration (P45, P9, W45, W9) and of pea and whey permeate at 10 mg P/ml concentration (Pperm10, Wperm10) was investigated in the Caco-2 cell system. The transepithelial electrical resistance (TEER) was measured after an incubation period of 1 h with test solution and after an additional hour of incubation with sodium fluorescein (Figure 4). TEER values are expressed as percent of the initial value, which was at least 300 $\Omega.cm^2$. For the control condition, where just transport buffer was added at the apical side during the first hour, the TEER decreased only slightly after 2 h at 37°C. The integrity of this cell monolayer was not compromised. Upon incubation with pea and whey digest at the highest concentration, hardly a stable TEER measurement could be obtained after 1 h and only about 20% of the initial TEER remained after the sodium fluorescein transport. This damage to the cell monolayer was also reflected in the sodium fluorescein flux, which was substantially higher for the 45 mg P/ml pea and whey digest than the value of 0.40% h^{-1} for the control, and even higher than the reference of 1% h^{-1} (Figure 4). Sodium fluorescein is a hydrophilic compound, which is supposed to cross the cell monolayer via the paracellular route with typical fluxes lower than 1% h^{-1} (Augustijns *et al.*, 1998). Acceptable transport validation parameters were obtained for 9 mg P/ml pea and whey digest and 10 mg P/ml pea and whey permeate. The IC_{50} values of the donor samples were comparable with the values mentioned in Figure 2. Hence, at the start of the experiment the ACE inhibitory activity at the apical side was 100% in all samples. When the basolateral samples were analysed immediately after the transport experiment, the 45 mg P/ml whey digest was the only sample for which significant ACE inhibitory activity was detected (Figure 5). Concentrating the control resulted in an apparent higher, although still non-significant, ACE inhibitory activity, which can be explained by a higher chloride concentration than the optimal one needed for maximal ACE activity (Chapter 1). Substantial ACE inhibitory activity was found in the concentrated basolateral samples for the 45 mg P/ml pea and whey digests conditions. The digests at the lower concentration and the whey permeate at 10 mg P/ml did not result in ACE inhibitory activity of the basolateral samples, while for the 10 mg P/ml pea permeate little ACE inhibitory activity was observed.

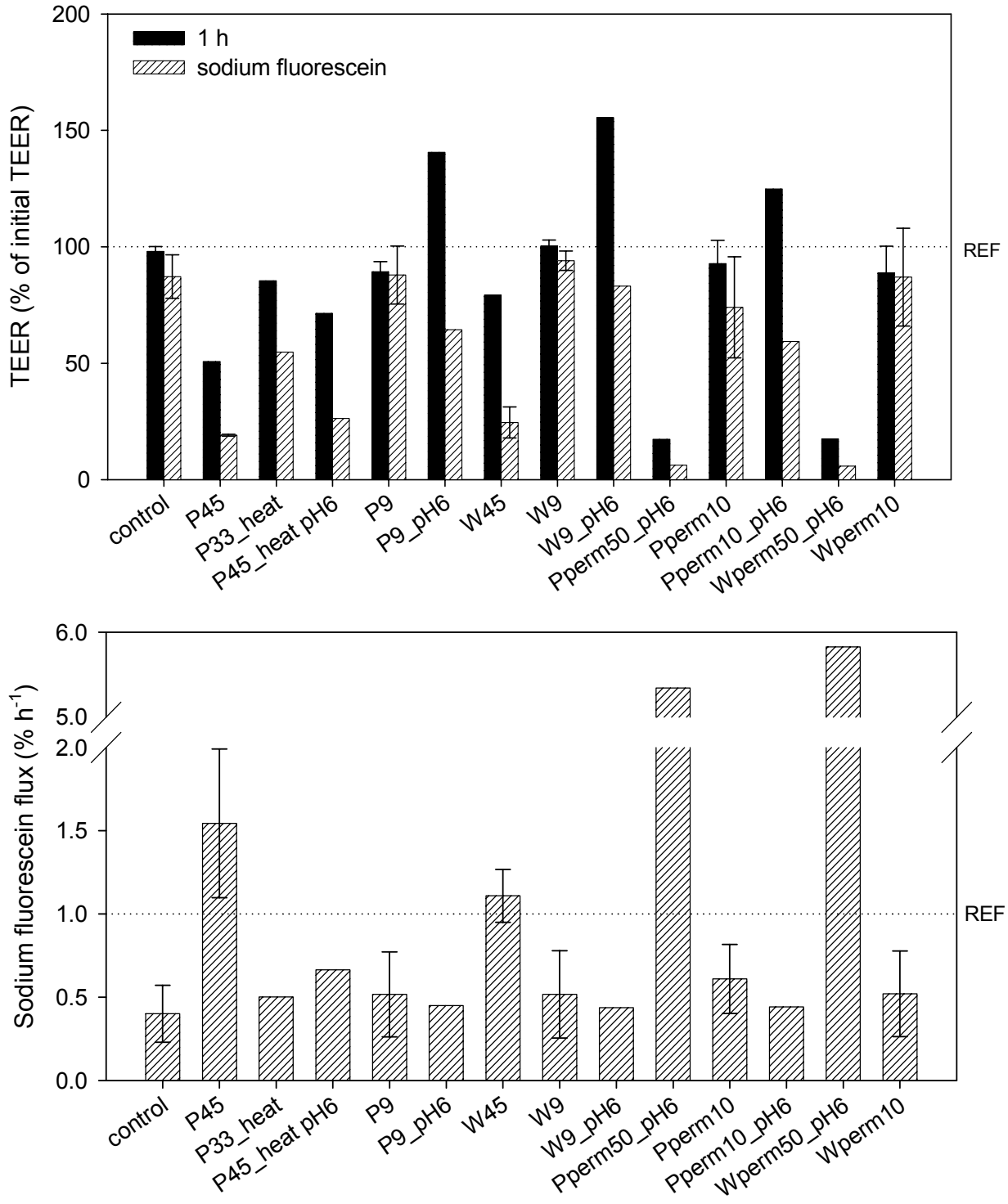


Figure 4. Transport validation parameters: TEER (%), after a 1 h incubation with test solution and after an additional 1 h incubation with sodium fluorescein solution; and sodium fluorescein flux (% h^{-1}). P, W, Pperm and Wperm: pea and whey digest and permeate respectively, the number is the concentration in mg P/ml, heat: 5 min at 98°C, pH 6: apical transport medium at pH 6 (single and repeated experiments).

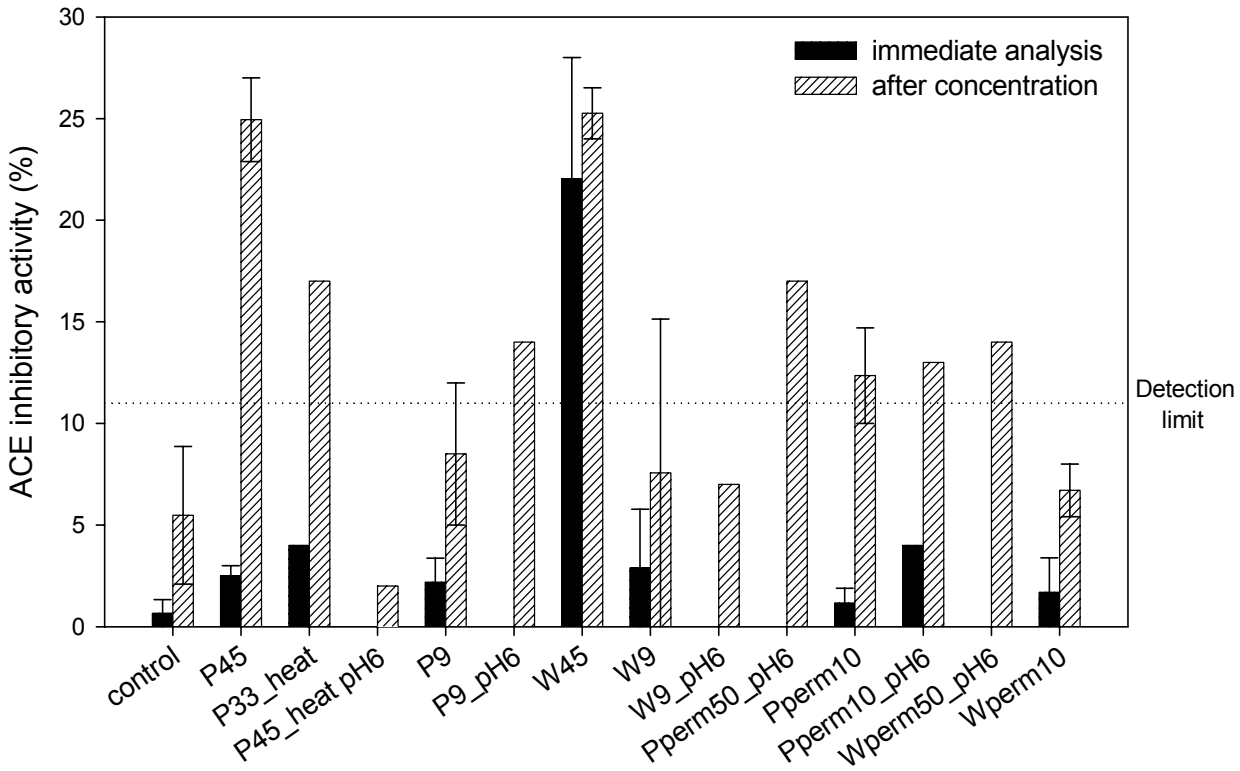


Figure 5. ACE inhibitory activity (%) in the basolateral compartment upon immediate analysis and in 4 times concentrated samples (no immediate analysis and 3 times concentrated in P45_heat pH6, Pperm50_pH6 and Wperm50_pH6). P, W, Pperm and Wperm: pea and whey digest and permeate respectively, the number is the concentration in mg P/ml, heat: 5 min at 98°C, pH 6: apical transport medium at pH 6 (single and repeated experiments).

In a second set of experiments, the pea digest at high concentration was heat treated (P33_heat) and 45 mg P/ml heat treated pea digest (P45_heat pH6), 9 mg P/ml pea and whey digest (P9_pH6, W9_pH6), 50 mg P/ml pea and whey permeate (Pperm50_pH6, Wperm50_pH6) and 10 mg P/ml pea permeate (Pperm10_pH6) were dissolved in transport buffer at pH 6. The transport of all these samples was studied in the Caco-2 model. Heat treatment did not affect the IC_{50} of the digest. The heated pea digests at high concentrations still showed substantial decreases in TEER values, but the sodium fluorescein fluxes were lower than the reference of 1 % h^{-1} (Figure 4). In the Caco-2 cell monolayer of pea and whey digest at 9 mg P/ml and pea permeate at 10 mg P/ml at pH 6, a substantial increase in TEER after 1 h incubation was observed. After fluorescein transport, the TEER value had substantially declined for the pea samples, while the TEER value of the 9 mg P/ml whey digest at pH 6 was at the level of the control sample. After incubation of the pea and whey permeate at 50 mg P/ml in the Caco-2 system, cell detachment could be observed. The harmfull effects of these samples were reflected in the TEER values that decreased to 6% at

the end of the experiment and the very high sodium fluorescein fluxes. ACE inhibitory activity was only observed after concentrating the basolateral samples (Figure 5). Lower ACE inhibitory activity was noticed in the heat treated pea digests compared to the untreated 45 mg P/ml pea digest. Pea digest at 9 mg P/ml and pea permeate at 10 mg P/ml at pH 6 had little, but significant ACE inhibitory activity after concentration, while whey digest at 9 mg P/ml at pH 6 showed no activity. The concentrated basolateral samples of 50 mg P/ml pea and whey permeate at pH 6 demonstrated significant ACE inhibitory activity as well.

The osmolarity of some donor samples was determined (Table 2). As an isotonic 9 g/l NaCl solution exhibits an osmolarity of about 300 mOsm, all these solutions, except the pea and whey digest at a concentration of 9 mg P/ml at pH 6, were hypertonic.

Table 2. Osmolarity (mOsm) of some donor samples in different concentrations.

	Pea	Whey
Digest 45 mg P/ml	573	472
Heat treated 33 mg P/ml	466	/
Digest 9 mg P/ml pH 6	377	353
Permeate 50 mg P/ml	1417	1498
Permeate 30 mg P/ml	957	1063
Permeate 10 mg P/ml	522	533
Permeate 10 mg P/ml pH 6	524	544

/ = not determined.

SHR experiments

To investigate the biological activity of the ACE inhibitory pea and whey permeates *in vivo*, doses of 50 mg P/kg BW were injected into the femoral vein of SHR. Physiological saline in an equal volume and captopril (5 mg/kg BW) served as negative and positive control, respectively. The evolution of the mean arterial blood pressure during 15 min from the start of the injection is depicted in Figure 6. The maximal drop in blood pressure occurred at different times in the different animals, partially due to variations in injection time. This caused less pronounced effects of the average over time. Therefore, the overall mean minimal blood pressure and blood pressure reduction ratio is mentioned in Table 3. In the control animals, the blood pressure remained at 141 ± 1 mmHg throughout the experiment.

Captopril exerted a significant blood pressure lowering effect from 40 s onwards, when on average the injection was completed, and reached a minimum level after 1 min 30 s. After 4 min the blood pressure increased again, showing in some rats the tendency to return to its initial value, while remaining at a reduced level in others, which explained the large standard error bars after 10 min. Captopril resulted in a maximal reduction ratio of 41%.

As the solutions of pea and whey permeate were more concentrated than the captopril and control test solutions, they were injected more slowly. Intravenous administration of pea permeate immediately caused a sharp and short blood pressure reduction. Only 20 and 25 s after the start of the injection the blood pressure was significantly lower than the start value, but this was largely due to variations in injection time. The maximal reduction ratio of pea permeate was 32%. At 2 min, when the total amount of pea permeate had been injected, the blood pressure increased above the initial value, but returned to it after 10 min. When whey permeate was injected in the femoral vein, a similar course in blood pressure could be observed, but the slight changes were non-significant.

The heart rate of the animals was not affected by any of the treatments.

Table 3. Initial and minimal mean arterial blood pressure (BP) (mmHg) and reduction ratio (%) after intravenous administration of captopril, pea and whey permeate.

	Initial BP	Minimal BP	Reduction ratio (%)
Captopril 5 mg/kg	141.0 ± 2.2	82.8 ± 1.1***	41
Pea permeate 50 mg P/kg	136.8 ± 4.2	92.4 ± 4.8***	32
Whey permeate 50 mg P/kg	140.0 ± 2.0	133.4 ± 2.5	/

Significantly different from the initial blood pressure : ***$p < 0.001$ ($n = 5$).
/ = not applicable.

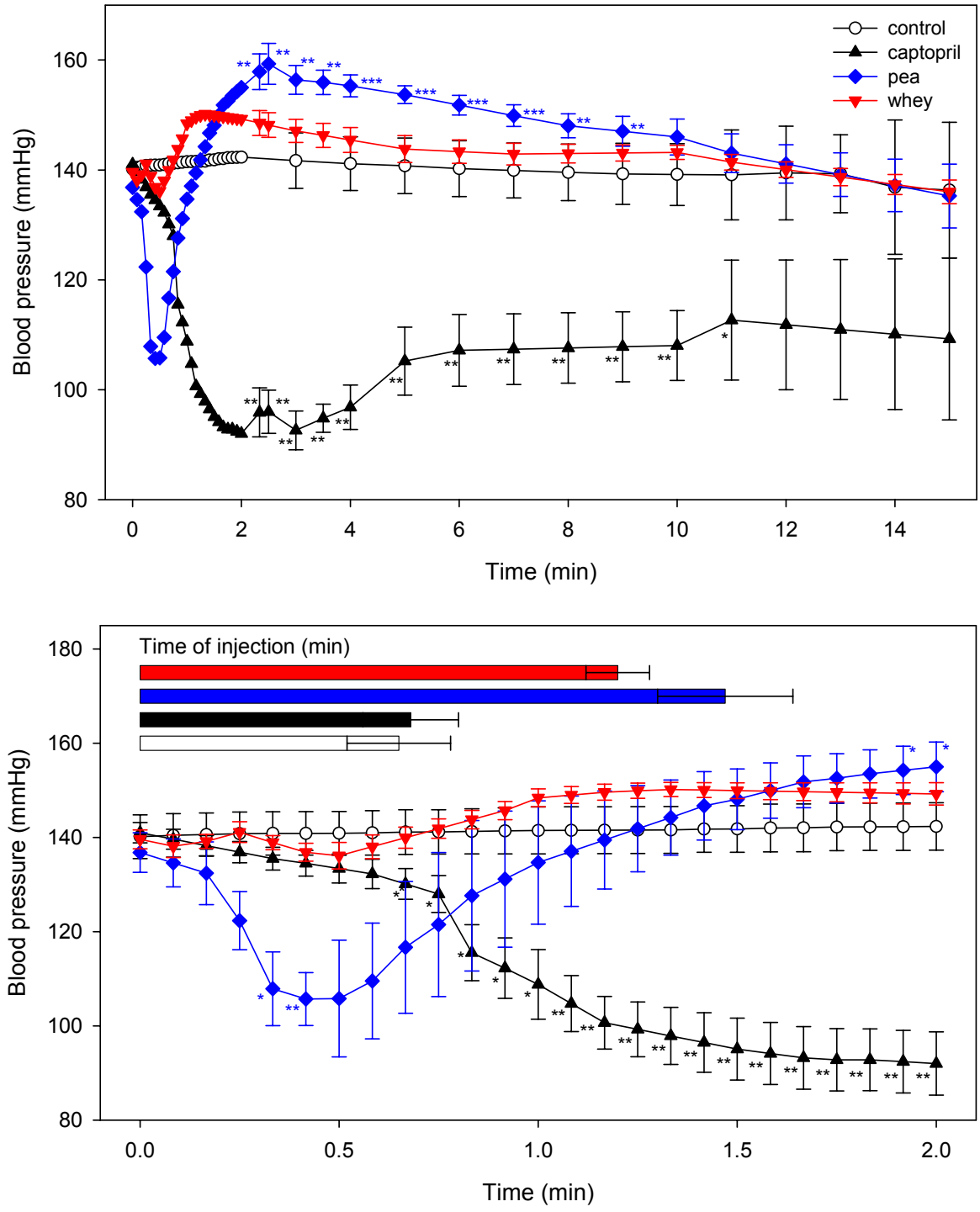


Figure 6. Evolution of the mean arterial blood pressure (mmHg) after injection of physiological saline (control), 5 mg/kg BW captopril, 50 mg P/kg BW pea permeate and 50 mg P/kg BW whey permeate during 15 min upon injection and during the first 2 min in detail (n = 5). The injection time (min) is also indicated.
*p < 0.05, **p < 0.01, ***p < 0.001 significantly different from the initial blood pressure.

DISCUSSION

Fractionation of pea and whey digests

Firstly, ultrafiltration was applied to pea and whey hydrolysates. This is a simple and reliable technique to separate peptides on molecular weight, while removing proteins. Moreover, it presents an effective sample preparation and clean-up technique for RP-HPLC (Herraiz, 1997). Bioactive peptides usually contain 2-20 amino acid residues per molecule (Pihlanto-Leppälä, 2001) and the lower their molecular weight, the higher their chance to cross the intestinal barrier and exert a biological effect (Roberts *et al.*, 1999). The MWCO in our study was 3000 Da, which corresponds to oligopeptides of about 25 amino acids. Ultrafiltration led to an enrichment of ACE inhibitory activity in the permeate of both pea and whey hydrolysate. In whey, the ACE inhibitory activity was even increased threefold by this treatment step. Compared to pea, whey hydrolysate seemed to contain a higher proportion of large peptides with lower ACE inhibitory activity. β-lactoglobulin, which is still partially present after *in vitro* gastrointestinal digestion as observed in Chapter 5 and 6, may bind low molecular weight ACE inhibitory peptides, preventing them from crossing the ultrafiltration membrane (Noiseux *et al.*, 2002). When fractionating ACE inhibitory peptides from whey hydrolysate, Pihlanto-Leppälä *et al.* (2000) found higher ACE inhibitory activity in the < 1 kDa fraction than in the other fractions. However, Mullally *et al.* (1997) reported that the permeates of tryptic digests of whey protein hydrolysates and β-lactoglobulin obtained after ultrafiltration through 3 kDa, as opposed to 1 kDa membranes, are more potent inhibitors of ACE. In a three-step recycling ultrafiltration membrane reactor, the gelatine hydrolysate fraction gained after the third hydrolysis with an ultrafiltration membrane of 1 kDa MWCO shows a higher ACE inhibitory activity than after the first and second hydrolysis steps with ultrafiltration membranes of 10 and 5 kDa respectively (Byun and Kim, 2001). Hence, our results confirm the observation that ultrafiltration may separate high ACE inhibitory activity.

Secondly, the permeates obtained by ultrafiltration-centrifugation were fractionated by RP-HPLC. This separates the peptides by molecular weight and hydrophobicity. The highest ACE inhibitory activity was found in fraction IV for pea and fraction III for whey, which corresponded with a gradient of about 24-28% acetonitrile. Hence, these fractions contained probably hydrophobic peptides, which are more likely to exert an ACE inhibitory effect according to the structure-activity relationship of ACE inhibitory peptides (Fitzgerald and Meisel, 2000). The first fractions showed a lower ACE inhibitory activity than the permeates and digests they originated from. Most likely, this can be attributed to the presence of amino acids and short hydrophilic peptides with low ACE inhibitory activity. The treatment was again more effective for whey than for pea. This may suggest that in whey hydrolysate very

potent ACE inhibitory peptides were present next to low active peptides, while in pea digest all peptides had more or less the same ACE inhibitory activity.

Caco-2 transport study

For the bioavailability of ACE inhibitory peptides, transport through the intestinal brush border forms a major issue, in addition to stability towards peptidases (Fitzgerald and Meisel, 2000). After addition of pea and whey digests or permeates to the apical compartment of a Caco-2 cell monolayer, no or only little ACE inhibitory activity was detected even in concentrated basolateral samples after 1 h at 37°C. It is very unlikely that this was caused by degradation by Caco-2 peptidases, as high ACE inhibitory activity was still recovered after 2 h incubation of pea and whey digests and permeates with Caco-2 homogenates at 37°C, although some decrease in activity could be observed. Whey digest and permeate seemed more susceptible to degradation by these peptidases than pea, or it can be that in pea digest and permeate ACE inhibitory peptides were broken down to other active peptides with the same total ACE inhibitory activity. This corresponds well with the observation in Chapter 6, where the ACE inhibitory activity of whey digest decreased more than the one of pea upon incubation with a rat intestinal acetone powder extract. Furthermore, this rules out the possibility that the change in ACE inhibitory activity in the digests in these experiments was solely due to the further action of the gastrointestinal proteases, as it also occurred in the permeates. In this respect, it was noticed that in a solution containing pepsin, trypsin and α-chymotrypsin in concentrations as supplemented during digestion, these enzymes were largely precipitated during the centrifugation step.

The intestinal transport of pea and whey digest at 45 and 9 mg P/ml concentration and of pea and whey permeate at 50 and 10 mg P/ml concentration was investigated in the Caco-2 cell model. These concentrations were first of all chosen to facilitate the detection of intestinal transport of active compounds. Similar concentrations to 10 mg P/ml or 5 mg P/well are reported in transport experiments in literature (Rubio and Seiquer, 2002; Winckler *et al.*, 1999). Considering that the ACE inhibitory peptides Val-Pro-Pro and Ile-Pro-Pro reach the intestinal brush border undegraded, consumption of 95 ml Calpis milk or 150 ml Evolus milk delivers respectively about 2.6 mg or 5.3 mg ACE inhibitory peptides to the intestinal barrier (Hata *et al.*, 1996; Seppo *et al.*, 2003). Hence, our lower concentrations are rather physiologically relevant.

There was a strong indication that the ACE inhibitory peptides in our samples were not transported through the Caco-2 cell monolayer, although the sensitivity of the ACE inhibition assay may have been too low to detect active compounds in the basolateral samples. The

45 mg P/ml digests and 50 mg P/ml permeates showed the highest ACE inhibitory activity in the basolateral compartments, but they compromised the integrity of the monolayer as indicated by the large decreases in TEER and the high sodium fluorescein transport. The hypertonicity of these solutions may be responsible for these harmful effects. The permeates showed a higher osmolarity than the digests at similar concentrations: due to the low molecular weight of the peptides in these samples, they contained at least the amount of solute particles present in the digests at the same protein concentration. Heat treatment alleviated the damaging of the cell monolayer and probably decreased the concentration of 'active solute particles' present. Despite having an osmolarity as high as the 45 mg P/ml digests, the 10 mg P/ml permeates did not evoke significant decreases in TEER or increased sodium fluorescein transport. As the osmolarity of the permeates was measured at a different point in time than that of the digests, this may be explained by a variation in the measurement. Another mechanism than hypertonicity may also have contributed to the harmful effects of the 45 mg P/ml digests. Applying transport buffer at pH 6 in the apical compartment did not result in increased transport of pea and whey digests and permeates. It has been shown that in the intestinal mucosa an acid microclimate region is present in the absorptive zone of the villus (Daniel and Herget, 1998). As a proton-motive force efficiently energises di- and tripeptide transport in the PepT1 transporter (Walker *et al.*, 1998), a lower mucosal pH may increase peptide uptake. *In vitro* studies with various tissue preparations reveal that there are only minor effects, if any, on peptide uptake when the pH of the incubation buffer is changed from neutral to acidic values. Hydrolysis of dipeptides appears to be reduced more significantly than the transport is increased. The Caco-2 cell monolayer however, lacks a three-dimensional structure and different cell types and may therefore not have a microclimate pH that differs significantly from bulk-phase pH (Daniel and Herget, 1998). Therefore lowering of the pH in the mucosal bathing solution results in an increased uptake of di- and tripeptides by PepT1 in the Caco-2 model (Winckler *et al.*, 1999; Yang *et al.*, 1999). As this effect was not observed, ACE inhibitory peptides may not present a substrate for the PepT1 transporter. When studying the transport mechanism of the ACE inhibitory peptide Val-Pro-Pro, addition of a competitive substrate for PepT1 or endocytosis inhibitors, like Gly-Pro, does not inhibit the transport of intact tripeptide across Caco-2 cell monolayers (Satake *et al.*, 2002). Although Val-Pro-Pro is composed out of hydrophobic amino acids, a peptide with only three amino acid residues may not be hydrophobic enough to be transported by passive diffusion or endocytosis. In order to cross the epithelial barrier transcellularly, a peptide must both have a low hydrogen bonding potential and a favourable octanol-water partition coefficient (Burton *et al.*, 1996). Therefore the paracellular route is suggested as the major pathway for intact transport of Val-Pro-Pro. This bioactive peptide has a flux of less than 2% h^{-1} in the Caco-2 cell monolayer, although it has been shown to be

present in the abdominal aorta and exerts an antihypertensive effect after oral intake in SHR (Masuda *et al.*, 1996). In another paper, where the transepithelial transport of bioactive oligopeptides in Caco-2 cell monolayers is investigated, susceptibility to peptidases is observed to be one of the primary factors that determine transport (Shimizu *et al.*, 1997). Adsorptive transcytosis, depending on the hydrophobicity of the peptides, is suggested to be involved in the transport of bradykinin, while the tetrapeptide Gly-Gly-Tyr-Arg would be transported mainly via the paracellular pathway.

Paracellular transport is regulated by the tight junctions, which are associated with a cytoskeletal structure and are therefore influenced by various factors that affect cellular structure and function (Nusrat *et al.*, 2000). During rapid fluid absorption (solvent drag) associated with sodium coupled transport of hexoses and amino acids in the small intestine, the tight junctions become dilated and allow the passage of solutes with theoretical molecular weight up to 1000 Da or more between absorptive cells. It has been shown that undegradable, mainly hydrophilic oligopeptides are transported in this way in substantial quantities (Pappenheimer *et al.*, 1997). The tight junction permeability can be estimated by measuring the TEER. As the resistance of the paracellular route is much lower than that of the transcellular one, the TEER across the epithelial monolayers reflects mainly the resistance offered by the tight junctions. Specific food ingredients, like extracts of sweet pepper, horse radish and some mushrooms, have demonstrated to decrease the TEER and hence increase the paracellular permeability in Caco-2 cell monolayers, without affecting the viability of the cells (Hashimoto *et al.*, 1994; Shimizu, 1999). By the same mechanism, sodium salts of medium-chained fatty acids derived from dairy products, sodium caprate (C10) in particular, can promote the intestinal absorption of undegradable oligopeptides without causing detrimental alterations of the intestinal mucosa (Chao *et al.*, 1999). Moreover, Caco-2 cell monolayers are known to be tighter than mammalian intestinal tissues (Boisset *et al.*, 2000). These findings suggest that the amount of bioactive peptides paracellularly absorbed during food intake *in vivo* could be higher than expected from the results of *in vitro* transport experiments. As the ACE inhibitory activity in our digests and permeates was relatively resistant towards peptidase activity, it is still possible that the bioactive peptides are transported *in vivo* in sufficient quantities to exert an antihypertensive effect.

SHR experiments

The intravenous administration experiments in SHR were validated by the use of physiological saline and captopril as negative and positive controls respectively. Captopril, as antihypertensive drug, exerted a strong reduction in blood pressure that was maintained for

several minutes. A similar observation is made by French *et al.* (1995), where after intravenous administration of captopril at a dose of 5 mg/kg BW to conscious SHR, the blood pressure, monitored in the carotid artery, has returned to the baseline level after 60 min. A reduction in the mean arterial blood pressure of 17 mmHg is obtained after 30 min. In another study, intravenous injection of captopril at a dose of 5 mg/kg BW immediately lowers the systolic blood pressure, monitored in the carotid artery, in SHR. Twenty minutes after injection a reduction of 75 mmHg is obtained and maintained for another 40 min till the end of the experiment (Shin *et al.*, 2001). However, it is difficult to compare the change in systolic blood pressure with the change in mean arterial blood pressure, as these are influenced by blood pressure-lowering agents differently (Fuglsang *et al.*, 2002).

Pea permeate at a dose of 50 mg/kg BW showed a transient and sharp antihypertensive effect that was immediately followed by an increase in blood pressure above the initial value. Later on, the blood pressure returned to the initial level. Whey permeate tended to follow the same course, but no significant changes were observed. These are both mixtures of unknown peptides dissolved in physiological saline at concentrations of about 30 mg P/ml. Permeates dissolved in transport buffer at this concentration were hypertonic. Hence, one possible explanation for the change in blood pressure observed could be the administration of hypertonic solutions. However, these solutions were injected slowly and injection of a hypertonic saline solution of about 410 mOsm in the femoral artery did not change the blood pressure (data not shown). Moreover, there was a substantial difference in the effect between pea and whey permeate. A more plausible clarification for the combination of an increase and a decrease in blood pressure would be the presence of both vasodilating or ACE inhibitory peptides and vasoconstricting peptides in the permeates. In addition to the ACE inhibition, food derived peptides may exert an antihypertensive effect by other mechanisms. From albumin, for example, two peptides have been isolated with vasorelaxing properties (Fujita *et al.*, 1995; Matoba *et al.*, 1999). Finally, the increase in blood pressure following the decrease may be explained by an unknown compensation mechanism in the animal. Peptides are rapidly degraded in plasma and some of them have half-lives in the order of minutes and even seconds (Gardner, 1998; Moskowitz, 2003). In this respect, the ACE inhibitory peptides in pea permeate, showing a higher stability to peptidases than whey permeate, might exert a transient antihypertensive effect before being broken down in the plasma.

In only one other study a mixture of peptides is intravenously administered to SHR. When wheat germ hydrolysate, dissolved in physiological saline, is injected at a dose of 50 mg/kg BW in the tail vein of SHR, a short decrease in mean arterial blood pressure, monitored in the carotid artery, of only 5% is obtained 13 min after injection (Matsui *et al.*, 2000). The

injection of Ile-Val-Tyr, a potent ACE inhibitory peptide isolated from wheat germ hydrolysate, at 5 mg/kg BW results in a more pronounced transient antihypertensive effect of 10% after 8 min. This peptide is found to be metabolised in plasma to form a subsequent ACE inhibitor, Val-Tyr. The absence of an antihypertensive effect after intravenous administration of ACE inhibitory peptides cannot exclude a protective role of these peptides in the long term or at a higher dose after single administration. For example, when a soy protein hydrolysate is fed daily to SHR, a dose of 100 mg/kg BW decreases the systolic blood pressure of SHR only after 12 days (Wu and Ding, 2001). Oligopeptides of 1 kDa or less, obtained from chicken egg yolk hydrolysate, suppress the development of hypertension in SHR after daily oral administration at 20 mg/kg BW for 12 weeks (Yoshii *et al.*, 2001).

Further research needs to elucidate the biological effects of pea and whey permeate *in vivo*.

In conclusion, the ACE inhibitory activity of pea and whey hydrolysates could be increased upon purification by ultrafiltration-centrifugation and RP-HPLC. When examining the intestinal transport of active compounds in pea and whey digests and permeates, only minor ACE inhibitory activity was observed to cross the Caco-2 cell monolayer. After intravenous administration in SHR, pea permeate exerted an antihypertensive effect that merits further research.

ACKNOWLEDGEMENTS

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CHAPTER 8

GENERAL DISCUSSION, CONCLUSIONS AND PERSPECTIVES

Chapter 8

General discussion, conclusions and perspectives

PROBLEM STATEMENT AND RELEVANCE OF THIS WORK

Hypertension is a significant public health problem worldwide that is associated with an increased risk for coronary heart disease, congestive heart failure, stroke and renal disease. It is estimated that 20% of the world's adult population suffers from hypertension. The incidence of hypertension becomes more prevalent with age and hypertension is found in about 65% of individuals above 65 years in many industrialised countries (Alper *et al.*, 2001). Moreover, the World Health Organisation (WHO) attributes 30% of all global deaths in 1998 to cardiovascular diseases (CVD) (WHO, 1999). In Belgium, the share of cardiovascular diseases in mortality is even bigger (Figure 1).

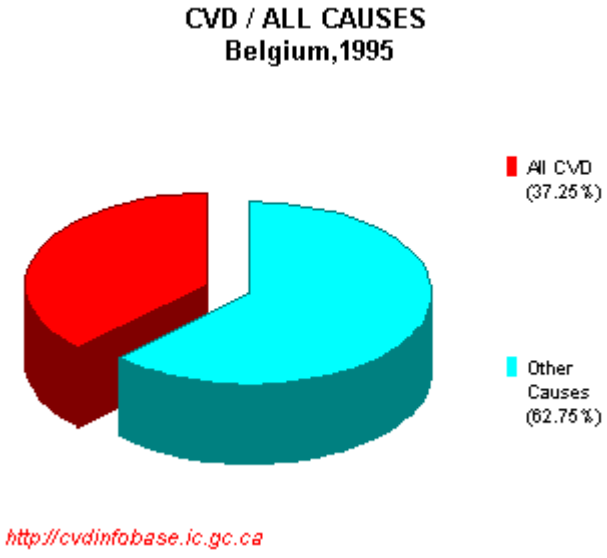

Figure 1. Proportional mortality of cardiovascular diseases (CVD) in Belgium, 1995 (Country profile for diseases of the circulatory system, www.who.org).

Lifestyle modifications and diet therapy are one of the most important tools for effective lowering of blood pressure (Hermansen, 2000). Moreover, even small decreases in blood pressure result in significantly lower risks for cardiovascular diseases (Van der Niepen, 2000). A higher dietary protein intake seems to have favourable influences on the blood

pressure in hypertensive individuals. Also the Dietary Approaches to Stop Hypertension trial (DASH), a diet rich in fruits, vegetables and low-fat dairy products, is associated with an effective reduction of the blood pressure (Hermansen, 2000). Although other mechanisms may play a role, ACE inhibition by bioactive peptide release from food proteins may have caused these antihypertensive effects. Indeed, a number of research reports have demonstrated the antihypertensive effect of ACE inhibitory peptides or foods containing these bioactive compounds in hypertensive patients, even when they received antihypertensive medication (Hata *et al.*, 1996; Itakura *et al.*, 2001; Pins and Keenan, 2002; Seppo *et al.*, 2002; Seppo *et al.*, 2003). In addition, several studies in spontaneously hypertensive rats (SHR) suggest a significant suppression of the development of hypertension upon receiving a diet rich in ACE inhibitory peptides (Sipola *et al.*, 2002; Yoshii *et al.*, 2001). Overall, this points out to the fact that **ACE inhibitory peptides, as part of a food product or as nutraceutical, may be of functional interest in both the treatment and the prevention of hypertension**. Compared to ACE inhibitory drugs, food derived peptides have certain advantages. As they usually have a lower activity and specificity, they do not demonstrate any side effects. They also represent a much lower cost to health care. As part of the daily diet, they appear more natural and safe to the consumer. However, ACE inhibitory drugs have proven their usefulness and it is not the intention to completely replace them, but in a number of cases, especially in the prevention of hypertension and as initial treatment in mild hypertensive individuals, food derived ACE inhibitory peptides could be applied, while in others they could function as an additional treatment.

Food derived ACE inhibitory peptides fit well in the new nutrition concept that emphasises the relation between nutrition and human health. The market for functional foods is increasing. According to a study from Business Communications Company, Inc., RGA-109 Functional/Nutraceutical/Welness Foods and Beverages, the sales of functional foods and beverages marketed in the US is estimated at $20 per person in 1999 (Rajan, 2000). Growing at an average annual growth rate of 12.8%, it is projected that the total market will increase to $36 per person in 2004. The larger share will be for functional beverages, although a faster growth is projected for functional snacks and meal items. However, defining the market for functional foods has proven problematic for market analysts since it is highly fragmented and covers a multitude of products with proven and unproven benefits. In this respect, functional foods are defined as those foods that are marketed on a health benefit and it does not necessarily include natural foods containing bioactive compounds.

Some functional foods containing ACE inhibitory peptides are already on the market. Calpis™ sour milk from The Calpis Food Industry Co., Ltd. (Tokyo, Japan) bears the FOSHU label in Japan (Takano, 2002) and is patented (Nakamura and Takano, 1995). Evolus® from Valio Ltd. (Helsinki, Finland) is a similar milk fermented by *Lactobacillus helveticus* LBK-16 H, but this strain is still alive in the fermented milk, the concentrations of the two ACE inhibitory tripeptides Val-Pro-Pro and Ile-Pro-Pro are doubled and the milk has a favourable electrolyte composition (Seppo *et al.*, 2003; Sipola *et al.*, 2002). Davisco Foods International, Inc. (Eden Prairie, MN, USA) brought BioZate® 1, a hydrolysed whey protein isolate supplement, on the market in the USA (Pins and Keenan, 2002). Katsuo-bushi oligopeptide, a thermolysin digest of dried bonito has also been approved as FOSHU in Japan (Fujita and Yoshikawa, 1999).

Figure 2. Functional foods containing ACE inhibitory peptides on the market.

Although ACE inhibitory peptides have been characterised from various food proteins from vegetable or animal origin, **pea protein has never been investigated**. Pea is a long established and significant crop in Europe and has some advantages for human nutrition. Pea protein has a well balanced profile of amino acids, and especially a high content in the essential amino acid lysine. Moreover, in accordance with today's consumer demands, it is environment-friendly produced: it may not only present a non-GMO alternative to soy protein, but it is a low input crop due to symbiotic nitrogen fixation (Schneider and Lacampagne, 2000). Therefore, pea protein was the subject of our study and was compared to whey protein, a high valuable protein, which originates as by-product from milk during the cheese making process.

This work aimed to study the formation of ACE inhibitory activity from pea and whey protein and to investigate *in vitro* the maintenance of this activity after oral administration in the human body, from the gastrointestinal tract to the site of action, the cardiovascular system. Compared to most ACE inhibitory peptide studies, where emphasis is put on the isolation of peptides, their *in vitro* ACE inhibitory activity and *in vivo* antihypertensive effect in SHR, this thesis stressed the importance of the gastrointestinal

proteases and peptidases in the formation and degradation of ACE inhibitory peptides and the need for intestinal transport of intact peptides. Gastrointestinal digestion and intestinal transport are the major barriers in the bioavailability of ACE inhibitory peptides (Pihlanto-Leppälä, 2001). We chose not to isolate one or a few peptides with high ACE inhibitory activity. In protein hydrolysates, the ACE inhibition measured is the result of various peptides, while the nutritional value of the protein is preserved. Moreover, it is suggested that peptide mixtures may exert a synergistic ACE inhibitory effect (Gobbetti *et al.*, 2000).

An overview of **the major accomplishments of this work** is given in Figure 3. In brief:

- An ACE inhibition assay was developed, optimised and validated (Chapter 2).
- Ala-Leu-Pro-Met-His-Ile-Arg, one of the most potent ACE inhibitory peptides isolated from whey protein up to date, resisted *in vitro* gastrointestinal digestion and was transported intact through a Caco-2 Bbe cell monolayer. However, this peptide was degraded in the presence of rat intestinal tissue and failed to reduce the blood pressure after intravenous administration in SHR at a dose of 5 mg/kg (Chapter 3).
- Fermentation by lactic acid bacteria and the yeast *Saccharomyces cerevisiae* was rather ineffective in releasing ACE inhibitory activity from pea and whey protein. *In vitro* gastrointestinal digestion seemed to be the predominant factor controlling the formation of ACE inhibitory activity from these food proteins (Chapter 4).
- *In vitro* gastrointestinal digestion of pea and whey protein released high ACE inhibitory activity. Comparison of three different *in vitro* digestions in batch, revealed that a digestion simulating the physiological conditions of protein hydrolysis was sufficient to obtain maximal ACE inhibitory activity. In a semi-continuous reactor model of the gastrointestinal digestion of pea protein, response surface methodology indicated that maximal ACE inhibitory activity was achieved at the longest incubation times in the stomach and small intestine phase within the model (Chapter 5).
- A time course study of the *in vitro* gastrointestinal digestion observed for pea protein high ACE inhibitory activity already in the stomach phase, while high ACE inhibitory activity was only attained in the small intestine phase for whey protein. SDS-PAGE showed that the major part of the proteins in pea was already broken down after the stomach phase digestion, while the major whey protein β-lactoglobulin was only degraded from the small intestine phase onwards. Supplementation of a rat intestinal acetone extract, simulating the intestinal brush border phase digestion, slightly decreased the ACE inhibitory activity and more for whey than pea digest (Chapter 6).

- An ACE inhibitory peptide database predicted very high ACE inhibitory peptide potency for β-lactoglobulin, that exceeded all other pea and whey proteins, and even above β-casein, a known source of potent ACE inhibitory peptides. *In silico* gastrointestinal digestion of the pea and whey proteins with the highest potencies characterised the release of ACE inhibitory peptide sequences (Chapter 6).
- The ACE inhibitory activity of pea and whey digest could be enriched by ultrafiltration-centrifugation and subsequent RP-HPLC fractionation of the permeate. Larger differences were obtained for whey than pea protein (Chapter 7).
- Although high ACE inhibitory activity was maintained after incubation of pea and whey digest and permeate with Caco-2 homogenates, no or only little ACE inhibitory activity was transported through Caco-2 cell monolayers (Chapter 7).
- After intravenous administration of a dose of 50 mg P/kg BW in SHR, pea permeate exerted a transient, but strong antihypertensive effect. Whey permeate exerted no effect at a dose of 50 mg P/kg BW (Chapter 7).

The importance of our findings is briefly discussed in the following section.

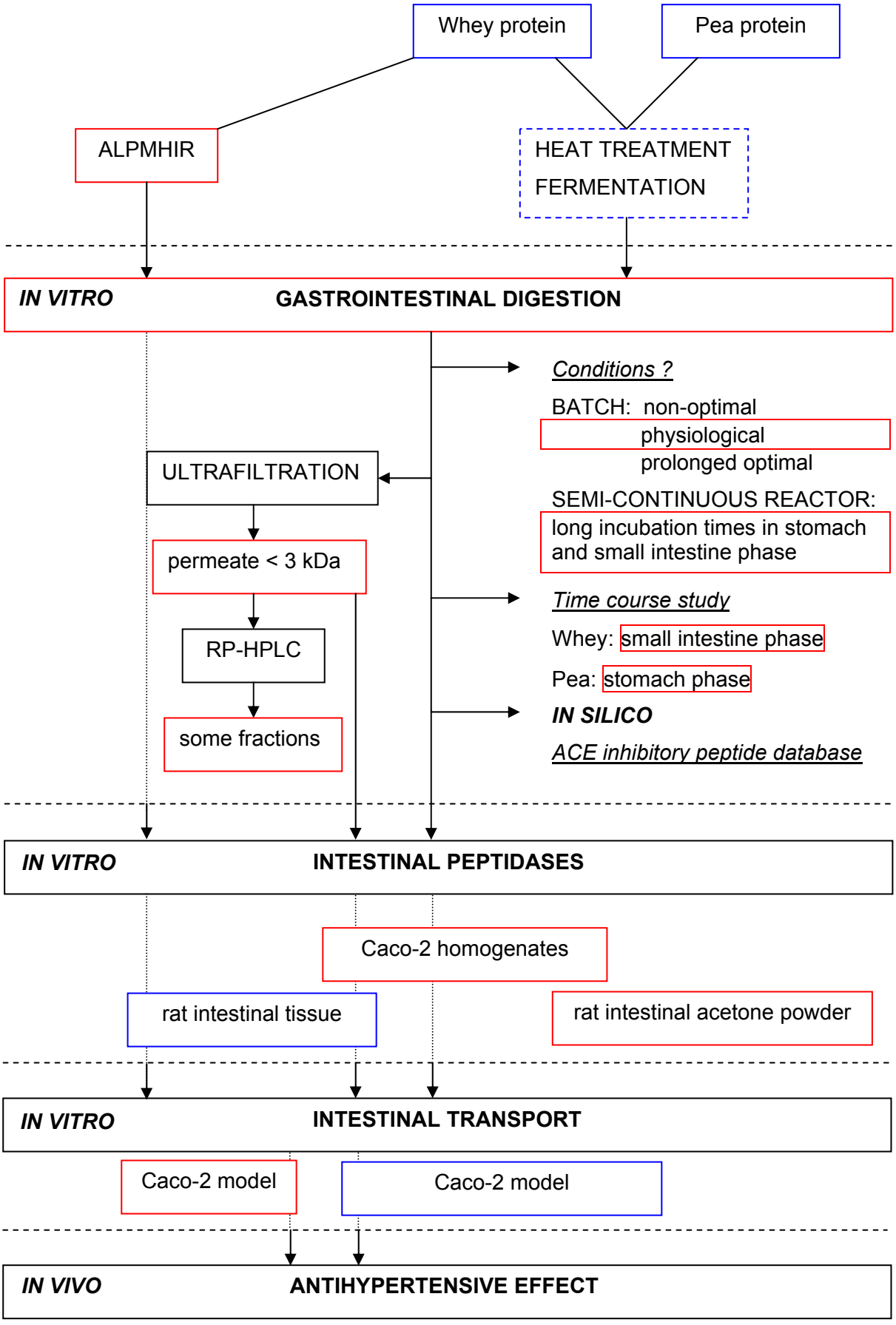

Figure 3. Schematic overview of the major accomplishments of this work. Red: high(er), blue: low(er) ACE inhibitory activity as determined by the ACE inhibition assay.

GENERAL DISCUSSION AND CONCLUSIONS

In vitro models

The maintenance of ACE inhibitory activity derived from pea and whey protein after oral administration in the human body was investigated by a combination of different *in vitro* models. *In vitro* models are commonly used as alternatives in the screening procedure of bioactive compounds, whether derived from food or plants or chemically synthesised. They contribute to the 3R concept of Replace, Reduce and Refine experiments with animals and humans. *In vitro* models have several advantages over *in vivo* experiments with laboratory animals and clinical trials in humans: they pose no ethical constraints, are more reproducible and suited for standardised operation, have lower costs, allow fast screening of numerous samples and are easier to investigate underlying mechanisms. The drawbacks inherent to these models are that they are less representative than the *in vivo* situation and that no conclusions can be made in the long term. All *in vitro* models have limitations and these need to be recognised. *In vitro* models are only useful if they are well validated so that a reliable predictive value is obtained. More sophisticated models may increase the similarity with the *in vivo* situation, but will be more complex at the same time. Depending on the goal of the research, the appropriate *in vitro* model should be selected.

ACE inhibitory activity *in vitro*

In this work, an ACE inhibition assay was developed from a diagnostic ACE activity test, further optimised and validated. It provided a more rapid and straightforward measurement of the ACE inhibitory activity compared to the widely used method of Cushman & Cheung and derivatives. Both methods may result in a slight overestimation of the ACE inhibitory activity of food protein hydrolysates, as peptide substrates of ACE also display ACE inhibitory activity in the assay. This can be circumvented on the pure peptide level by measuring the ACE inhibitory activity before and after pre-incubation of the peptide with ACE, where the ACE of the pre-incubation needs to be inactivated first, and a follow up of the degradation of the peptide by pre-incubation with ACE on HPLC (Fujita *et al.*, 2000). As we focussed on food peptide mixtures, this approach was far too difficult for the purpose of our research and we just took this limitation into account. In literature, the majority of the ACE inhibitory peptide studies never mention this fact. Substrates for ACE are anyhow eliminated in the oral delivery route: if they already resist the gastrointestinal proteases, they are either degraded by ACE in the intestinal brush border membrane or by ACE in the plasma. The small increase in IC_{50} observed upon incubation of pea and whey digest with rat intestinal acetone

powder may have been partially due to substrates of ACE that were degraded. As the effect was more pronounced for pea than whey, it could be speculated that more substrates for ACE were present in the whey compared to the pea digests. Nevertheless, still high ACE inhibitory activity was found after this hydrolysis phase, indicating that a substantial amount of real ACE inhibitors was present in the digests. Further optimisation suggestions for the ACE inhibition assay are decreasing the sample volume of the inhibitory sample, which could be very useful in profound fractionation and isolation of ACE inhibitory peptides, and transforming the test to a multi-well plate assay, which will make the measurement even less labour intensive. A similar ACE inhibition assay, using FAPGG as substrate and measured in 96 well plates during 15 min at 340 nm, was recently mentioned in a study of van der Ven *et al.* (2002).

Antihypertensive effect *in vivo*

It is difficult to establish a direct relation between the ACE inhibitory activity *in vitro* and the antihypertensive activity *in vivo***.** Not only does the bioavailability after oral administration play a major role, but also other antihypertensive mechanisms than ACE inhibition may be of interest e.g. in the case of multifunctional peptides with additional opiate or vasorelaxing properties. In addition, different ACE inhibition assays, where other substrates and calculations for the IC_{50} are used, hamper the comparison of ACE inhibitory activities. Also in *in vivo* experiments and clinical trials, different experimental designs (measurement of the mean arterial or systolic and/or diastolic blood pressure; intravenous, subcutaneous or oral administration; other doses) and the use of the SHR model versus the hypertensive patient, hinder stating an unequivocal effect. Although the IC_{50} of captopril is about thousand fold lower than that of food derived ACE inhibitory peptides, there is not such a discrepancy observed in the antihypertensive effect. Indeed, oral administration of 200 mg/kg BW dipeptide isolated from garlic results in a reduction of blood pressure in SHR of about 30 mmHg, while captopril at a dose of 10 mg/kg BW exerts an antihypertensive effect of about 50 mmHg (Suetsuna, 1998). There are no substantial differences in duration of the effect. When compared on molar basis, the antihypertensive activity of Leu-Lys-Pro after oral and intravenous administration in SHR, amounted to 91% and 20% respectively to that of captopril, whereas the *in vitro* ACE inhibitory activity was 7.73% compared to captopril (Fujita and Yoshikawa, 1999). Hence, these findings indicate that compared to antihypertensive drugs, food derived ACE inhibitory peptides with antihypertensive activity possess higher *in vivo* activity than what would be expected from their *in vitro* activity. A sound explanation for this observation cannot be proposed yet, but in addition to the reasons mentioned above, it is suggested that food derived peptides have higher affinities for tissues and are more slowly

eliminated than the synthetic captopril (Fujita and Yoshikawa, 1999). Moreover, it is plausible that the access to the ACE enzyme *in vivo* is limited so that above a certain affinity substances inhibit ACE *in vivo* to a similar extent. After feeding of a soy protein hydrolysate at 100 mg/kg BW in SHR during one month, the blood pressure decreased by 38 mmHg, compared to 46 mmHg for 50 mg/kg BW captopril (Wu and Ding, 2001). When assessing the ACE activity in serum, aorta and lung of these rats, the soy ACE inhibitory peptides did not show any significant effect compared to the control, whereas captopril greatly enhanced the ACE activity in serum and reduced the ACE activity in the aorta. Although the real causes of these observations remain unknown, these different responses might indicate that food derived ACE inhibitory peptides and captopril act via different antihypertensive mechanisms.

Another remarkable effect is that an antihypertensive effect of ACE inhibitory peptides is only detected in SHR and hypertensive individuals and not in Wistar Kyoto rats (WKY) and normotensive individuals (Itakura *et al.*, 2001; Nakamura *et al.*, 1995b). This is directly related to their mechanism of action, because the opiate peptide α-lactorphin, although it has some ACE inhibitory activity, lowered the blood pressure in both SHR and WKY and this effect could be abolished by naloxone, a specific opiate receptor antagonist (Nurminen *et al.*, 2000).

Hence, further investigation into the antihypertensive effect of food derived ACE inhibitory peptides is necessary. Yet, the showing of *in vitro* ACE inhibitory activity is a good starting point, because it is based on a biological mechanism. As ACE exhibits several functions in the human body, ACE inhibition may have additional or other implications than antihypertensive effects (Moskowitz, 2002).

Intestinal transport

Few studies on the transport of ACE inhibitory peptides have been undertaken in literature, although it is important for the bioavailability of these peptides to investigate if they are transported and what the transport mechanism is. The Caco-2 cell monolayer was used to investigate intestinal transport in this work. This confluent cell monolayer displays several properties typical for differentiated intestinal epithelial cells (Wilson *et al.*, 1990) and is widely used as model in transport studies of drugs (Augustijns *et al.*, 1998; Boisset *et al.*, 2000) and food compounds (Rubio and Seiquer, 2002). Caco-2 cell monolayers are known to be tighter than mammalian intestinal tissues (Boisset *et al.*, 2000), while brush border membrane associated enzyme activities are generally lower (Bolte *et al.*, 1998). Species-specific differences in intestinal brush border enzyme activity have been observed (Drucker *et al.*, 1997). For example, esterase activities in homogenates prepared from freshly scraped

intestinal mucosa of rat is up to six times higher than that of pig, the latter considered more representative for the human small intestine (Augustijns *et al.*, 1998). In order to get a total overview of the transport possibilities and limitations of a certain compound, it is advised to apply different absorption models. Considering the information above, Ala-Leu-Pro-Met-His-Ile-Arg may be degraded in the human intestine to a lesser extent than in the rat intestinal tissue homogenates and it may be transported in higher concentrations than observed in the Caco-2 Bbe cell monolayer. Likewise, pea and whey digests and permeates may be absorbed *in vivo*, although in the Caco-2 model almost no transport of their ACE inhibitory activity is observed. Moreover, the digests and permeates have the advantage of showing resistance to degradation by intestinal peptidases from Caco-2 homogenates and rat intestinal acetone powder. It is believed that resistance to the brush border peptidases is the predominant factor for transport. Besides, during oral consumption of ACE inhibitory peptides, the presence of other food compounds may have important consequences on the susceptibility to peptidase degradation and intestinal transport (Charman *et al.*, 1997). While some specific food compounds have been observed to decrease the TEER in the Caco-2 cell monolayer (Shimizu, 1999), active transport of sugars and amino acids initiates a solvent drag through the tight junctions in rat intestine, by which oligopeptides may be absorbed (Pappenheimer and Volpp, 1992). This could be an additional advantage of protein hydrolysates containing amino acids, compared to pure peptides.

Pea versus whey protein

In our study, pea and whey protein demonstrated some different characteristics, which can be related to their amino acid sequence and protein structure. Although pea protein was more susceptible to gastrointestinal proteases than whey, the latter had a lower IC_{50} value after *in vitro* physiological digestion, but this was only reached after the small intestine phase. After enrichment of ACE inhibitory peptides from the digests by ultrafiltration-centrifugation and RP-HPLC, the increases in ACE inhibitory activity were more substantial in whey compared to pea. While pea digest seemed to contain peptides with more or less similar ACE inhibitory activity, in whey digest potent ACE inhibitory peptides were present next to low active peptides. Combining SDS-PAGE and RP-HPLC experimental data with theoretical data from an ACE inhibitory peptide database and data-mining program revealed that β-lactoglobulin, the main whey protein, was responsible for these phenomena. This protein exhibited high ACE inhibitory peptide potential and resisted digestion by pepsin. The different pea proteins on the other hand were all rather easily digested and showed similar ACE inhibitory potential, which was lower than β-lactoglobulin. Moreover, *in silico* gastrointestinal digestion directly released Ala-Leu-Pro-Met-His-Ile-Arg from β-lactoglobulin, which could also

be released in the *in vitro* experiments. To our knowledge, this is the first study that applied a large ACE inhibitory peptide database to predict potentials of protein sequences. Such a database may prove to be particularly useful in nutrition science: instead of a 'trial and error' approach where ACE inhibitory peptides can be isolated from food proteins by applying certain enzymes, a food protein knowing to contain a lot of potent ACE inhibitory peptide sequences may be chosen to be digested by an enzyme that specifically will release these bioactive fragments.

Pea digest and permeate exhibited higher ACE inhibitory activity than whey upon incubation with rat intestinal acetone powder or Caco-2 homogenates, due to a higher resistance towards brush border peptidases. The susceptibility to plasma peptidases may also have been the reason why pea permeate exerted a certain antihypertensive effect upon intravenous administration in SHR, while whey permeate was ineffective. However, further SHR experiments such as the daily oral administration of different doses over a longer period of time, need to further substantiate the possible antihypertensive effect of pea and whey permeate or digest.

This thesis indicates some important physiological implications for the release of ACE inhibitory activity *in vivo* during gastrointestinal digestion of pea and whey protein. In healthy subjects maximal ACE inhibitory activity release after gastrointestinal digestion of pea and whey protein can be expected. In older persons that have a higher non-optimal pH in the stomach (Morihara *et al.*, 2001), it is suggested that considerably lower ACE inhibitory activity is released, even more for whey than pea protein. In this respect, a production process based on the gastrointestinal digestion, like in the semi-continuous reactor model, has the advantage that potent ACE inhibitory hydrolysates that resist the physiological digestion, are orally administered. The results obtained by response surface modelling indicate that factors that prolonge the residence time in stomach and small intestine may enhance the release of ACE inhibitory activity. Examples are protein-rich, fat and hyperosmolalic food and sensations of fear (Ganong, 1997a). In this respect, peptides present in a food matrix may react with other food components, which can influence both the activity and the bioavailability of ACE inhibitory peptides. This, together with the effect of processing conditions, needs to be investigated when developing a food process technology for ACE inhibitory products.

PERSPECTIVES

New approaches for production and bioavailability of ACE inhibitory peptides

In addition to the exploration of new proteins in this field, a more purposeful approach is the screening of the protein sequence, if available, against an **ACE inhibitory peptide database** as described in Chapter 6. Hence, proteins with high ACE inhibitory peptide potential can be selected a priori and specific enzymes that will release the active sequences can be chosen.

ACE inhibitory peptides may be chemically synthesised based on known peptide sequences. Although high costs and low quantities are generally associated with this production process, recent advantages in the **design and construction of synthetic peptides** make this approach more attractive. Standard solid-phase approaches using fluorenyl-methoxycarbonyl (FMOC) chemistry can synthesise routinely peptides to lengths of about 40-50 amino acids. Chemical ligation, the chemoselective reaction of unprotected peptide segments, is a more novel approach that has made the total synthesis of longer polypeptides possible even in water, at a pH of 7 (Muir *et al.*, 1997). At present, one is able to design peptides with specific folding motifs to obtain more thermodynamically stable molecules and even more interestingly, to design peptides with specific functions. To accomplish this, one must first have an understanding of the spatial relationship amongst residues that will promote the desired activity. This usually requires knowledge of the X-ray crystal or NMR solution structure of the protein or peptide and deriving structure-activity relationships (SARs). For ACE inhibitory peptides, mostly short sequences without a tertiary structure, only the latter is of importance. SARs are generally developed by substituting residues in the native sequence and assessing the subsequent effects on structure and activity. Bioactivities of short peptides can be optimised by using combinatorial libraries (Dooley and Houghten, 1999; Mayo, 2000). A more detailed knowledge on the structure-activity relationship of ACE inhibitory peptides and the crystallisation and mechanism of action of ACE will both contribute to and benefit from this approach. In addition, it may be investigated if amino acids that resist proteolysis *in vivo*, e.g. D-amino acids, can be incorporated with the maintenance of biological activity. Computer programs are also available that investigate the permeability of peptides (Brayden and O'Mahony, 1998). Finally, in this way an ACE inhibitory peptide database can be created and extended.

Some peptides, however, are still difficult to chemically synthesise. **Genetic engineering** may represent an alternative tool to produce these and other ACE inhibitory peptides in large amounts. The chemical synthesis of DNA provides the opportunity to construct and modify

natural genes, thereby making it possible to create an artificial gene coding for a specific ACE inhibitory peptide or a combination of peptides. However, in order to be applied as functional food or nutraceutical, these peptides must be produced with a high yield by recombinant DNA technology. Recently, the expression and purification of an ACE inhibitory peptide multimer in *Escherichia coli* containing the ACE inhibitory peptides Ile-Tyr and Val-Lys-Tyr, was described (Oh *et al.*, 2002). Artificial genes were designed based on the oligonucleotide sequence of the decapeptide Asp-Phe-Ile-Tyr-Val-Lys-Tyr-Gly-Pro-Gly. Degenerative codons for amino acids were used to avoid limitation of supply. The presence of Asp-Phe can counteract the positive charge of lysine, providing a more stable and less toxic peptide in the host cell. Phenylalanine and tyrosine make the peptide cleavable by α-chymotrypsin for the release of the ACE inhibitory peptide sequences. Two ligated repeated sequences were cloned into a plasmid vector and after isolation of the fragment it was recloned in pAP1, a plasmid vector already containing the synthetic DNA fragment. This step was repeated once more to end up with clones containing genes encoding up to 6 repeats of the decapeptide, pAP3. Hence, the sequence was long enough to study the expression of the artificial protein. After isolation of this DNA fragment, it was cloned into another plasmid that was used to transform an *E. coli* strain. In this way, the artifical protein PA3 was expressed as a fusion protein, GST-AP3-6His. After recovering the inclusion bodies from the cells, the protein was easily purified with affinity chromatography on a glutathione sepharose column, due to the His-tag. The resin-bound fusion protein was cleaved with thrombin and the peptide AP3-6His was further purified on RP-HPLC. A yield of 7.9% of this oligopeptide was obtained after purification. After α-chymotrypsin hydrolysis and fractionation on RP-HPLC, two potent ACE inhibitory peptide fractions containing the peptides Ile-Tyr and Val-Lys-Tyr could be isolated. They constituted approximately 35% of the total AP3-hydrolysate.

Several expression vector systems are commercially available to yield substantial amounts of designed proteins. Most of them, however, make the subsequent purification of the artificial protein necessary because the expressed protein resides in the host cell, as described in the above study. In the same way as therapeutic compounds are delivered *in situ* (Krüger *et al.*, 2002; Steidler, 2002), ACE inhibitory propeptides could be delivered via microorganisms to the small intestine, where they could be expressed and exported outside the cell, cleaved into active peptides by the action of proteases and/or peptidases and finally transported into the blood stream. A GRAS microorganism that is able to survive the gastrointestinal transit and has additional probiotic properties, could be selected. This bacterium should be genetically engineered to express upon induction the ACE inhibitory propeptide, coupled to a signal peptide to direct the translocation of the protein through the

cell membrane. An inducible promoter should increase ACE inhibitory peptide release in the small intestine and prevent the expression of the polypeptide in the large intestine, where it is of no use and could eventually lead to the formation of harmful nitrogen compounds. When genetically modified organisms (GMO) are more understood and accepted, a functional food based on this concept might have great value, because it not only would enhance the oral delivery of ACE inhibitory peptides, but it also would enable the combination of different functional effects in one food. In addition to microorganisms, genetical engineering may also be applied to plants and animals, where modified proteins, rich in ACE inhibitory peptide sequences, could be obtained.

Despite their susceptibility to metabolisation and sometimes low membrane permeability, peptides have been recognised as important therapeutic compounds and therefore several approaches have been designed to **increase the oral delivery of peptides**. In addition to *in situ* delivery and modification of the peptide to obtain higher stability, the bioavailability of ACE inhibitory peptides may be increased by cross linking the target peptide to protein transduction domains or by means of specific peptide carriers like Pep-1 that are able to deliver biologically active proteins and peptides into mammalian cells (Morris *et al.*, 2001). Peptide permeation may also be achieved by chemical enhancers and surfactant-like agents, provided the tissue recovers *in vivo* after temporary exposure. Receptor targeting and mucoadhesion represent some other strategies that may increase the delivery of peptides (Brayden and O'Mahony, 1998).

ACE affinity chromatography was applied to isolate an ACE binding protein of about 14 kDa from human serum (Thevananther and Brecher, 1999). This technology might also be further explored for a more straightforward fractionation of ACE inhibitory peptides. A detailed knowledge about the structures of the active domains of ACE would allow coating columns with smaller particles for a specific methodology.

ACE has more in store

The literature review already pointed out to the many different functions of the ACE enzyme. Moreover, relative overactivity of ACE (D/D genotype) appears to be associated with many age-dependent common diseases in humans, including cardiovascular disease, cancer and psychiatric disease (Moskowitz, 2002). ACE inhibitors are common treatments for direct hypertension related diseases like congestive heart failure, myocardial infarction, diabetes, chronic renal insufficiency and atherosclerotic cardiovascular diseases. However, hypertension is also significantly associated with cerebrovascular disease, neoplasms, endocrine disorders, gastrointestinal diseases, psychiatric disorders, dementia, skin

problems and blood diseases (Bunk, 2002). Besides, the successful application of ACE inhibitors in a number of these diseases suggests that ACE is an early rate-limiting step for multiple disease pathways (Figure 4). Since ACE inhibitors have superior, although not yet fully explained, clinically efficacy and an established safety profile, many additional disease targets might benefit from the administration of ACE inhibitors (Moskowitz, 2002). The broad functionality of ACE also increases the application possibility of food derived ACE inhibitory peptides.

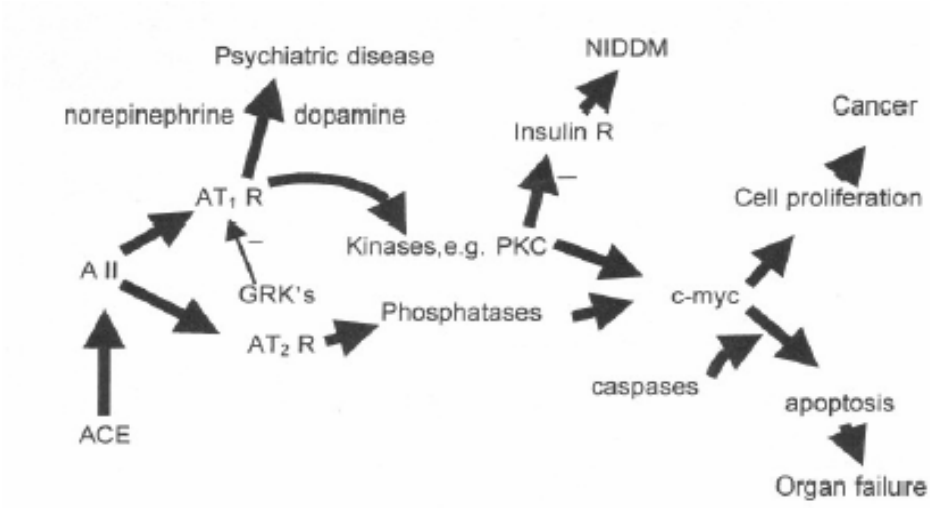

Figure 4. ACE at the start of a disease pathway of common diseases (Moskowitz, 2002).

Recently, a model has been designed for the active site of ACE and the functional and structural differences in the C- and N-domain (Moskowitz, 2003). The ancestral ACE gene underwent duplication at the origin of Chordata, at the same time that the circulation became closed, resulting in increased systemic pressure. Moskowitz (2003) suggests that the duplicated ACE, 'somatic' ACE, functions as a mechanosensor, defending downstream vessels and tissues from an increase in pressure. In this model, ACE senses shear stress (blood velocity) on vessel walls in regions of turbulent blood flow (Figure 5). This occurs in plaque containing regions or when cholesterol and fatty acids thicken membrane fluidity, so that ACE spins too slowly to orient itself in the direction of the blood flow. Both N- and C-domains have an evolutionary conserved tripeptide Phe-X-Pro that is positioned near the active site and contains a charged middle amino acid in the C-terminal and a neutral middle amino acid in the N-terminal domain. An increase in shear stress strips this autoinhibitor tripeptide, Phe-Gln-Pro, from the N-terminal active site, thereby activating it. The C-terminal domain is constitutively activated by chloride. Crystallisation of the duplicated holoenzyme must validate this hypothesis.

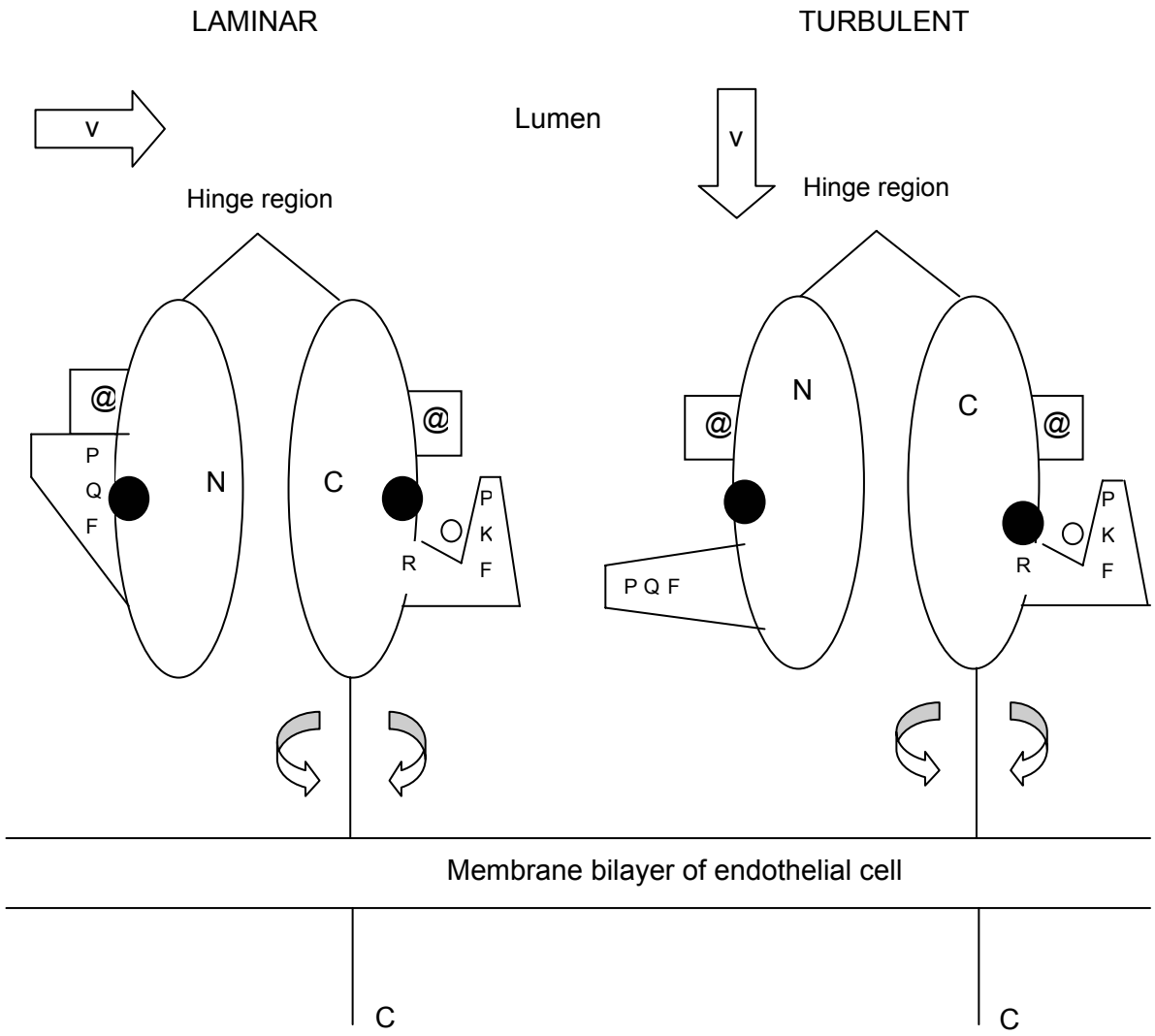

Figure 5. Model of somatic ACE with the active C- and N-domains under laminar blood flow (left) and turbulent blood flow (right) conditions. Arrow with v = blood velocity vector, solid circle = zinc ion, open circle = chloride ion, @ = stop feature at active site preventing protrusion of more than two or three amino acids past the active site, N = N-terminal domain, C = C-terminal domain, R, P, K, Q, F = one letter amino acid code. The C-terminal active site has been opened by chloride ion. The N-terminal active site is occluded in a region of laminar blood flow. In a region of turbulent blood flow, the N-terminal active site is opened by a mechanical shearing force (Moskowitz, 2003).

Hence, the N-terminal active domain of ACE may regulate ACE *in vivo*. Moskowitz (2003) proposes that the systemic blood pressure may be regulated by the C-domain, while local signals may be due to the activation of the N-terminal active site, e.g. target organ damage.

This model also explains the clinical superiority of hydrophobic ACE inhibitors relative to hydrophilic ones. All ACE inhibitors bind to C-terminal sites, but only hydrophobic ones like captopril, quinapril and ramipril bind the occluded N-site and are therefore better at blocking

angiotensin II production. Moreover, it can explain why these inhibitors have specific local benefits in addition to a reduction in the systemic blood pressure (Moskowitz, 2003).

This new information may have important implications for food derived ACE inhibitory peptides as well. Based on their structure, hydrophobic or hydrophilic, they will show a different inhibition of the ACE enzyme, resulting in different effects in the human body.

Systems biology will change nutrition research

In applying biomarkers in human studies, the relationship between nutritional intervention, the biomarkers and the supposed health effect, in many instances, has not been straightforward. This is also the case for the relation of ACE inhibitory activity *in vitro* and an antihypertensive effect *in vivo*. New biological methods can offer new perspectives in this respect. Genomics, genotyping, transcriptomics, proteomics and metabolomics, together with bioinformatics, constitute the discipline of functional genomics, which is also referred to as systems biology. Instead of focussing on a single gene or protein only, systems biology studies the whole tissue, organ or organism. In theory, the a priori knowledge of the molecular mechanism is not necessary, but will be revealed from the complex dataset. The integration of systems biology into nutrition research is termed nutritional genomics or nutrigenomics (van Ommen and Stierum, 2002; Verrips *et al.*, 2001). This describes a new approach to food and nutrition research, in which these powerful functional genomic technologies are used in combination with established nutritional and biochemical techniques to determine the mechanisms by which foods and their individual components modulate processes occurring in the human body. Moreover, answers can be formulated on questions related to dose effects and individual requirements according to genetic profile, age, gender and lifestyle. Individual genetic differences in response to dietary components have been evident for years: lactose intolerance, alcohol dehydrogenase deficiency, individual and population differences in blood lipid profiles and health outcomes after consumption of high fat diets.

In the respect of our work, the expression of hypertension related genes may be screened with several ACE inhibitory peptides to reveal the overall mechanisms by which ACE inhibitory peptides exert effects. This methodology may also provide answers to what roles gender, ethnicity and age play in ACE activity and its inhibition. Furthermore, the successful application of ACE inhibitors in a number of diseases may be mechanistically explained.

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ADDENDUM

Addendum

Composition of Pisane® (Cosucra), pea protein isolate and Lacprodan® DI-9213 (Acatris), whey protein isolate

Component	Content (% of dry matter) *(UTI/mg dry matter)	
	PEA	WHEY
protein (N x 6.25)	90 ± 2	min. 90
fat (petroleum ether method)	0.2	max. 0.2
fibres	1.8	
starch	0.7	
sugars	1	max. 0.2
ash	max. 5 (mainly K, Na, P)	5 (mainly P)
water	max. 6	max. 5
anti-nutritional factors:		
α-galactosides	0.9	
trypsin inhibitors	1.25*	
phytic acid	1.5	
lectins	0	

Physical specifications

	PEA	WHEY
pH (5% solution)	6.8-7.5	2.8-3.5
density	0.33 g/cm^3	0.45 g/cm^3

Amino acid composition

Amino acid	Content (% of total nitrogen content)	
	PEA	WHEY
alanine	4.7	4.7
arginine	9.0	1.9
aspartate	12.3	11.0
cysteine	1.2	2.4
glutamate	19.8	16.7
glycine	4.6	1.7
histidine*	2.8	1.9
isoleucine*	4.6	6.1
leucine*	8.0	10.2
lysine*	8.2	9.2
methionine*	1.1	1.8
phenylalanine*	5.5	3.0
proline	4.5	5.8
serine	5.4	5.0
threonine*	4.1	7.3
tryptophan*	1.0	1.7
tyrosine	4.1	3.7
valine*	5.2	5.9

* dietary essential amino acids

CURRICULUM VITAE

Curriculum vitae

Name	Vanessa Vermeirssen
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E-mail	Vanessa.Vermeirssen@UGent.be
Place of birth	Dendermonde
Date of birth	January 15, 1977
Nationality	Belgian
Marital status	single
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EDUCATION AND PROFESSION

9/1988-6/1994	**Latin-mathematics**, Royal Atheneum of Dendermonde (greatest distinction, awards for mathematics and moral philosophy)
10/1994-6/1999	**Bio-engineer chemistry**, Faculty of Agricultural and Applied Biological Sciences, Ghent University (great distinction) Thesis: Enzymatic and microbial activities in the simulator of the gastrointestinal tract of the baby (baby-SHIME, in cooperation with P&G), Scientific promotor: prof. dr. ir. W. Verstraete, Laboratory of Microbial Ecology and Technology
10/1999-9/2002	**Doctoral training in Applied Biological Sciences**, Faculty of Agricultural and Applied Biological Sciences, Ghent University
10/1999-present	**FWO aspirant**, PhD grant of the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen, Faculty of Agricultural and Applied Biological Sciences, Ghent University Research: Release and activity of ACE inhibitory peptides from pea and whey protein, Scientific promotors: prof. dr. ir. W. Verstraete, Laboratory of Microbial Ecology and Technology and prof. dr. ir. J. Van Camp, Department of Food Technology and Nutrition

Collaborations: prof. dr. P. Augustijns, Laboratory of Pharmacotechnology and Biopharmacy, Catholic University of Leuven (KUL) and prof. dr. N. Morel, Laboratory of Pharmacology, Catholic University of Leuven (UCL) in Belgium; prof. dr. H.R. Gaskins, Department of Animal Sciences and prof. dr. K.A. Tappenden, Department of Food Science and Nutrition, University of Illinois, Urbana-Champaign in USA; dr. A. van der Bent, Institute for Agronomical and Technological Research (ATO) in The Netherlands

SCIENTIFIC OUTPUT

publications in peer reviewed journals

- P. De Boever, R. Wouters, V. Vermeirssen, N. Boon & W. Verstraete (2001). Development of a six-stage culture system for simulating the gastrointestinal microbiota of weaned infants. *Microbial Ecology in Health and Disease*, 13(2), 111-123.
- V. Vermeirssen, B. Deplancke, K.A. Tappenden, J. Van Camp, H.R. Gaskins & W. Verstraete (2002). Intestinal transport of the lactokinin Ala-Leu-Pro-Met-His-Ile-Arg through a Caco-2 Bbe monolayer. *Journal of Peptide Science*, 8(3), 95-100
- V. Vermeirssen, J. Van Camp & W. Verstraete (2002). Optimisation and validation of an angiotensin-converting enzyme inhibition assay for the screening of bioactive peptides. *Journal of Biochemical and Biophysical Methods*, 51(1), 75-87
- V. Vermeirssen, J. Van Camp, K. Decroos, L. Van Wijmelbeke & W. Verstraete (2003). The impact of fermentation and *in vitro* digestion on the formation of ACE inhibitory activity from pea and whey protein. *Journal of Dairy Science*, 86(2), 429-438.
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participation to conferences and workshops

- V. Vermeirssen & W. Verstraete (2000). *In vitro* simulation of the gastrointestinal tract. Workshop on microbial management in live food and larval fish production, Ghent University, Ghent, May 29, 2000. *Oral contribution*.

- V. Vermeirssen, J. Van Camp & W. Verstraete (2000). Angiotensin Converting Enzyme (ACE) inhibitory activity in pea protein ferments and hydrolysates. *Conference on Rumen Function*, 25, 40.
  Proceedings of 25th Conference on Rumen Function: 49 years of interaction, Chicago, Illinois, USA, November 14-16, 2000. *Poster presentation.*
- V. Vermeirssen & W. Verstraete (2000). Gastrointestinal microbiology and hygiene. 14th Forum of Applied Biotechnology (FAB), Bruges, September 27-28th, 2000. *Poster presentation.*
- V. Vermeirssen, B. Deplancke, K.A. Tappenden, J. Van Camp, H.R. Gaskins & W. Verstraete (2001). Intestinal transport of ACE inhibitory peptides derived from food proteins, studied with Caco-2Bbe monolayer in an Ussing Chamber experiment. *Journal of Physiology and Biochemistry*, 57 (2), 202.
  Proceedings of 17th Meeting of the European Intestinal Transport Group, S'Agaro [Girona], Spain, May, 5-8, 2001. *Oral contribution*.
- W. Maes, J. Van Camp, M. Hemeryck, V. Vermeirssen, J.-M. Ketelslegers, J. Schrezenmeir, P. Van Oostveldt & A. Huyghebaert (2001). Influence of the lactokinin Ala-Leu-Pro-Met-His-Ile-Arg on the release of endothelin-1 by porcine aortic endothelial cells. *Annuals of Nutrition and Metabolism*, 45(suppl. 1),140.
  Proceedings of 17th International Congress of Nutrition, Vienna, Austria, August, 27-31, 2001. *Poster presentation*.
- V. Vermeirssen, K. Decroos, L. Devos, J. Van Camp, P. Augustijns & W. Verstraete (2002). ACE inhibitory activity during *in vitro* gastrointestinal digestion and absorption of pea and whey protein. *American Journal of Clinical Nutrition*, 75(2), 426S.
  Proceedings of The First Annual Nutrition Week, San Diego, California, USA, February, 23-28, 2002. *Poster presentation.*
- V. Vermeirssen, J. Van Camp, P. Augustijns & W. Verstraete (2002). ACE inhibitory activity from pea and whey protein: *in vitro* gastrointestinal digestion and transport in Caco-2 model. 5de Voedings- en gezondheidscongres, Brussels, November, 15-16, 2002. *Poster presentation*.

publications in national journals without review

- V. Vermeirssen, T. Van De Wiele & W. Verstraete (2001). Development of laboratorium organs as an alternative to animal experiments. *In vitro* models of the gastrointestinal microbiota. 50th Post-universitaire onderwijsdag: de impact van de bio-ingenieur in de biomedische sector, Verhandelingen van de Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen, volume 41, 47-53.

Proceedings of 50ste Post-universitaire onderwijsdag, Gent, December 5, 2001. *Oral contribution.*

- V. Vermeirssen, J. Van Camp, P. Augustijns & W. Verstraete (2002). Angiotensin-I converting enzyme (ACE) inhibitory peptides derived from pea and whey protein. Mededelingen Landbouwkundige en Toegepaste Biologische Wetenschappen, 67(4), 27-30.
  Proceedings of 8th PhD Symposium on Applied Biological Sciences, Gent, October 9, 2002. *Oral contribution*.

projects

- Nieuw onderzoeksinitiatief Bijzonder Onderzoeksfonds Universiteit Gent 2003-2007: ACE and ACE inhibitory peptides in insects (prof. dr. ir. J. Van Camp and prof. dr. ir. G. Smagghe)

PRACTICAL TRAINING AND INTERNATIONAL EXPERIENCES

9/1996	IAAS-training (International Association of Agricultural Students) in Portugal: dairy cow production
9/1997	Microbiology project for P&G at v.z.w. Alitech (Ghent University, Department of Food Technology and Nutrition, Prof. dr. ir. Huyghebaert): scientific research into the microbial contamination/regrowth of stained and unstained clothing, washed with a detergent with or without amylase
9/1998	International course (Socrates): food packaging, Ghent; project: packaging of dehydrated foods
10/1998	Communicatiedagen KVIV: communication and presentation skills
28-29/11/1998	AISEC Case Study Weekend (P&G), Brussels: solving business questions taken from reality
1999-2002	Collaborator in the practical exercises for the bio-engineer courses: nutrition, partim gastrointestinal microbiota and *in vitro* intestinal transport (prof. J. Van Camp); microbial ecological processes (prof. W. Verstraete) Collaborator in the practical exercises for the GAS environmental sanitation course: environmental microbiology (prof. W. Verstraete)
1999-2002	Tutor of 3 bio-engineer students (Ghent University) and one graduated nutritionist (KHBO, Katholieke Hogeschool Brugge Oostende) during training and completion of their thesis

21/10-22/12/2000	Research at the University of Illinois, Department of Animal Sciences (Dr. H.R. Gaskins) and Department of Food Science and Nutrition (Dr. K.A. Tappenden), Urbana-Champaign, USA: *In vitro* intestinal transport and immunomodulatory properties of ACE inhibitory peptides. Travel allowance granted by Boehringer Ingelheim Fonds
2001-2002	Responsible at the laboratory for the Dionex HPLC system
22/04-6/05/2002	Start-up of the SHIME (Simulator of the Human Intestinal Microbial Ecosystem) apparatus at the Lab of Dr. Satya Prakash, Department of Biomedical Engineering, Faculty of Medicine, McGill University, Montréal, Canada